Molecular Characterisation of Penicillin-Resistant Isolates of *Streptococcus pneumoniae* in Christchurch, New Zealand.

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Abstract

Streptococcus pneumoniae is an important cause of morbidity and mortality worldwide. Acquisition of antimicrobial resistance, particularly to β-lactams, by this pathogen has complicated treatment options. The prevalence of penicillin-resistant pneumococci in New Zealand has increased greatly since 1993. To investigate the population structure of resistant pneumococci, 200 isolates collected between 1997-2001 with reduced susceptibility to penicillin were obtained from a Christchurch clinical laboratory. Isolates were examined using DNA macro-restriction profiling (MRP) and analysis of *pbp* genes. Four major clonal lineages were identified, the largest and most homogenous containing 97 (48.5%) of the isolates. Two of the four major PFGE groups (MRPs 2 and 3), could tentatively be described as belonging to globally widespread epidemic clones; the Spain^{23F}-1 clone and the France^{9V}-3 clone respectively.

To verify whether the New Zealand penicillin-resistant clones of *S. pneumoniae* had been recovered elsewhere in the world, 74 clinical isolates were chosen for analysis by multilocus sequence typing (MLST). These isolates were national samples, and represented pneumococci from multiple serotypes, multiple years, and from both invasive and non-invasive isolates. MLST is a technique based on nucleotide sequences of housekeeping genes. MLST permits strain identification by interrogation of a global database on the Internet. The isolates identified from throughout New Zealand were mainly found to belong to well-described international clonal lineages. The globally widespread Spain^{23F}-1 and Spain^{9V}-3 clones were represented by 14 and 11 isolates, respectively. The most prolific group of antibiotic-resistant pneumococci in New Zealand were derived from a recently described Taiwan^{19F}-14 clone, and represented by 26 isolates.

Although the Taiwan^{19F}-14 clone seemed genetically homogenous as assessed by MRP, the isolates varied in phenotype as observed by the range of β-lactam MICs within the clonal population. Examination of β-lactam resistance in New Zealand variant of the Taiwan^{19F}-14 clone was performed by *pbp* gene RFLP and DNA sequence analysis. Resistance was imparted at the molecular level by mosaic *pbp* genes. It appears that horizontal gene transfer from other resistant *S. pneumoniae* isolates has led to current *pbp* alleles observed in the Taiwan^{19F}-14 clone.

The molecular mechanism of erythromycin resistance was investigated in 150 pneumococcal isolates. On hundred and forty-one isolates (94.0%) had high-level erythromycin resistance (MIC ≥ 256 μg/ml); the remaining 9 (6.0%) isolates had MICs between 2-8 μg/ml. PCR detection of the macrolide resistance determinants was performed on all 150 isolates. The *mef*(A) gene was detected in 105 (70.0%) isolates and the *erm*(B) gene in 142 (94.7%) isolates. Both the *mef*(A) and *erm*(B) genes were detected in 97 (64.7%) isolates. The *mef*(A) *erm*(B) genotype was associated with the New Zealand variant of the Taiwan^{19F}-14 clone.

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This is for you.

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ACF	Abdominal cavity fluid
ATCC	American Type Culture Collection
bp	Base pairs
BLAST	Basic local alignment search tool
BHI	Brain-heart infusion
°C	Degrees centigrade
CFU	Colony forming units
CHEF	Clamped homologous electric field
CSF	Cerebral spinal fluid
CSP	Competence stimulating peptide
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example
ESR	Institute for Environmental and Scientific Research
EtBr	Ethidium bromide
g	Gram(s)
h	Hour(s)
HPLC	High performance liquid chromatography
IAA	Isoamyl alcohol
kb	Kilobase pairs (10^3 nucleotide bases)
kDa	Kilo Daltons
kJ	Kilo Joules
kPa	Kilo Pascals
KSC	Kenepuru science centre
M	Molar
mA	Milliamp
Mb	Megabase pairs (10^6 nucleotide bases)
MIC	Minimum inhibitory concentration
µg	Microgram(s)
µl	Microlitre(s)
min	Minute(s)
ml	Millilitre(s)
MLEE	Multilocus enzyme electrophoresis
MLRT	Multilocus restriction typing
MLST	Multilocus sequence typing
mm	Millimetre(s)

MRP	Macro-restriction profile
mth	Month(s)
nm	Nanometre(s)
NAM	N-acetylmuramic acid
NAG	N-acetylglucosamine
NCCLS	National Committee of Clinical Laboratory Standards
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDF	Peritoneal dialysis fluid
PFGE	Pulsed-field gel electrophoresis
PMEN	Pneumococcal molecular epidemiology network
RFLP	Restriction fragment length polymorphism
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
sec	Second(s)
ST	Sequence type
TEMED	N,N,N',N'-tetramethylethylenediamine
TIGR	The Institute for Genomic Research
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
TSA	Tryptic soya agar
UV	Ultra violet
v/v	Ratio of volume to volume
WHO	World Health Organisation
wk	Week(s)
x *g*	Times gravity

Chapter 1

Introduction

1.1 History of *Streptococcus pneumoniae*

The pneumococcus was first identified in 1881 concurrently by Louis Pasteur in France, who named it *Microbe septicèmique du salive*, and George Sternberg in the United States who called it *Micrococcus pasteuri*. By the late 1880s the organism had become widely recognised as the most common cause of lobar pneumonia and consequently adopted the name *pneumococcus*. The name *Diplococcus pneumoniae* was assigned in 1920, which was eventually to be changed to its current name, *Streptococcus pneumoniae*, in 1974. The term pneumococcus still perseveres today as a common term for *S. pneumoniae*, and the two are used interchangeably in this text. A comprehensive review of the early classification of *S. pneumoniae* is provided in Benajamin White's *Biology of Pneumococcus*, to which the interested reader is referred (White, 1938).

Few organisms have contributed as much information to the understanding of bacterial disease and the biology of the cell as the pneumococcus (Watson et al., 1993). For example, laboratory studies with *S. pneumoniae* led to the discovery of the first polysaccharide antigen, when previously it had been supposed only proteins were antigenic. This discovery is attributed to Heidelberger and Avery who showed in 1928 that the polysaccharide capsule of the pneumococcus was antigenic and responsible for its type specificity (Austrian, 1971, Watson et al., 1993).

S. pneumoniae played an important role in the discovery of DNA. Griffith in the 1920s challenged mice with an intraperitoneal injection of living, unencapsulated ("rough"), avirulent type II pneumococci together with heat killed, encapsulated ("smooth"), virulent type III pneumococci. The mice frequently developed a fatal infection. Culture of heart blood from dying animals recovered viable encapsulated type III pneumococci (Griffith, 1928). This observation was dubbed "transformation". The exact nature of the transforming agent responsible remained a mystery until the 1940s when Avery, Macleod and McCarty provided

evidence that DNA was in fact the "transforming principle" capable of restoring the ability to produce a capsule (Avery et al., 1944).

The history of discovery attributed to the pneumococcus, and the researchers who have worked with this organism since its discovery, have facilitated some of the most influential findings in biological science over the last century. The above list is far from exhaustive; the history of the pneumococcus in biomedical research has been subject of several comprehensive reviews (Austrian, 1971, 1981, Watson et al., 1993), which do this field far more justice than permitted herein.

1.2 Description of *S. pneumoniae*

The genus *Streptococcus* comprises a number of species from virulent pathogens to harmless commensals. Members of this genus are described as being Gram-positive, catalase-negative, facultative anaerobic bacteria that are spherical or ovoid and less than 2 μm in diameter, typically in pairs (Deibel and Seeley Jr, 1975). Streptococci are unable to synthesize haem-containing compounds and are consequently incapable of respiratory metabolism. Carbohydrates are metabolised fermentatively, with lactic acid being the main metabolic end product. Streptococci are nutritionally fastidious with variable and complex growth requirements. Blood or serum is often added to the culture medium to enhance growth. The growth of several streptococcal species (e.g., *S. pneumoniae, Streptococcus mitis*) is further stimulated in a CO_2 enriched (5%) atmosphere. Growth of streptococci on blood agar provides a useful means of differentiation based on their haemolytic properties. Haemolysis is defined as α-haemolysis (incomplete lysis and "greening" of agar), β-haemolysis (complete lysis of erythrocytes and clearing of agar) or χ-haemolysis (no lysis).

Many streptococci are human pathogens. *Streptococcus pyogenes*, the β-haemolytic, Lancefield group A streptococcus, is an etiological agent of many types of infection, including pharyngitis, respiratory infections, glomonephritis, skin and soft tissue infections, endocarditis, meningitis and scarlet fever. *S. pyogenes* is a strictly human pathogen, spread from person to person via respiration, or saliva droplets. The β-haemolytic, Lancefield group B streptococcus, *Streptococcus algactiae*, is an important cause of neonatal infection, characterised by sepsis and meningitis. Lancefield groups C and G are β-haemolytic streptococci, and produce a range of infections similar to *S. pyogenes*.

Like the β-haemolytic streptococci, *S. pneumoniae* is also pathogenic to humans. However, by 16S rDNA analysis *S. pneumoniae* is found to fall within the "mitis" group streptococci, whereas most of the other pathogenic streptococci cluster together in the "pyogenic" group (Kawamura et al., 1995). The streptococci most closely related to the pneumococcus include *S. mitis* and *S. oralis*, both of which are components of the normal oral flora (Kawamura et al., 1995).

On blood agar, *S. pneumoniae* produces colonies of characteristic appearance. Colonies are approximately 1 mm in diameter and surrounded by a green zone of α-haemolysis. Pneumococci produce polysaccharide capsules, which give colonies a moist glistening appearance. Serotype 3 pneumococci are particularly mucoid, and the colonies look correspondingly larger. The pneumococcal capsule is antigenic and is the basis of immunological typing. There are currently 90 different pneumococcal serotypes recognised – the last six (serotypes 10B, 10C, 11D, 12B, 25A, and 33D) being recognised only recently (Henrichsen, 1995). Two nomenclature systems exist for pneumococcal serotypes. The Danish system that groups antigenically cross-reacting types into clusters, and the American system that lists serotypes in order of discovery. By comparison, the Danish serogroup 19 includes serotypes 19F, 19A, 19B and 19C (the letter F corresponds to the first member of the group to be identified, and is then followed A, B, C etc), under the American system these are types 19, 57, 58 and 59 (Lund, 1960). The Danish nomenclature is currently the preferred of the two systems (Paton and Morona, 2000). The pneumococcal capsule is approximately 200-400 nm thick, and is attached to the cell wall peptidoglycan (Alonso De Velasco et al., 1995). Structurally, the capsule consists of high molecular weight polymers comprised of repeating oligosaccharide units. The component oligosaccharide units vary in complexity from the very simple-linear polymers of two monosaccharides (such as the type 3 capsule) – or more complex branching chain polysaccharides (Paton and Morona, 2000). The nature of the capsule as a virulence factor is addressed in section 1.3.2.1.

S. pneumoniae has the ability to bind and take up large pieces of DNA from the environment by a process called natural genetic competence. Natural genetic competence occurs in a variety of bacterial species, including *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Bacillus subtilis* and *S. pneumoniae* (Solomon and Grossman, 1996). The uptake mechanism in the Gram-positive bacteria (*S. pneumoniae* and *B. subtilis*) are similar; DNA is bound without sequence specificity, the DNA undergoes double-stranded cleavage, one strand is taken into the cell while the other is degraded extracellularly (Dubnau and Provvedi, 2000). Slight differences have been

noted with Gram-negative bacteria, presumably due to the presence of an outer membrane. In most organisms natural genetic transformation is a regulated process (an exception being *N. gonorrhoeae* which is constitutively competent). In *S. pneumoniae*, cell-to-cell signalling induces competence. During exponential phase growth, the pneumococcus secretes a peptide pheromone. When this pheromone reaches a critical concentration in the culture media, competence is induced (Tomasz, 1966). The competence pheromone is a 17-residue oligopeptide, the product of the *comC* gene (Håvarstein et al., 1995).

Genome sequences of two pneumococcal strains have recently been made public; the complete genome sequence of the avirulent laboratory strain, R6, (Hoskins et al., 2001); and the incomplete (>90%) genome sequence of virulent serotype 19F clinical strain, G54, (Dopazo et al., 2001). The R6 and G54 genomes have been shown to be 2.038 and 2.066 Mbp in size respectively, both with 40% GC content. The pneumococcus R6 genome contains none of the 18 genes encoding enzymes that comprise the tricarboxylic acid cycle (Hoskins et al., 2001). As a result, *S. pneumoniae* R6 is incapable of synthesizing amino acids from TCA cycle intermediates. This is consistent with the nutritionally fastidious nature of the pneumococcus (Deibel and Seeley Jr, 1975). *S. pneumoniae* cannot synthesize aspartate, aspargine, lysine, methionine, threonine and isoleucine (ultimately derived from oxaloacetate) or glutamate and arginine (derived from α-ketoglutarate). Furthermore, complete pathways for the synthesis of glycine, histidine and leucine could not be identified with the genome. Not surprisingly, these amino acids are included within a defined chemical media specifically designed for the cultivation of the pneumococcus (Tomasz and Hotchkiss, 1964). Many genes for the synthesis of various cofactors are also absent. Scattered within the R6 genome are numerous genes that are apparently derived from other bacteria (Hoskins et al., 2001). Presumably this has occurred as a consequence of natural competence in the pneumococcus. There are 40 ORFs present similar to those found in Gram-negative bacteria and that have no known homolog in Gram-positive species. Furthermore, approximately 2% of the R6 genes are truncated (particularly at the 5' ends) relative to orthologous genes in other bacteria. This again could be attributed to promiscuous horizontal gene transfer in the pneumococcus. No less than 60 complete or partial IS elements were also found in the R6 genome, representing ten different IS families.

Determination of the genome sequence of at least one other pneumococcal strain is currently in progress. A serotype 4 strain, KNR.7/87 has been sequenced as an ongoing project initiated by The Institute of Genomic Research (TIGR). Although unfinished, TIGR have made the partial sequence accessible to researchers for some time (http://www.tigr.org).

A brief description is worthy of pneumococcal strain R36 and its derivatives. All nonencapsulated isolates currently used in genetic studies of *S. pneumoniae* are derived from a single type 2 clinical isolate (D39) and its nonencapsulated derivative, used in the 1944 studies of Avery and colleagues; strain R36 (Avery et al., 1944). This strain has been exhaustively studied since its initial isolation in 1928. Subsequently, many derivatives of this strain have been documented, including R36A, R6, Rx and Rx1. These strain names; particularly R6 and Rx1 are used throughout this work. For a more detailed ancestry of these strains, see descriptions of Yother (Yother, 2000).

1.3 *S. pneumoniae* : Host - Pathogen Interactions

1.3.1 Pathology of pneumococcal infections

S. pneumoniae is the leading cause of potentially life threatening community-acquired disease. In the United States the estimated annual number of pneumococcal infections includes 0.5 million cases of community acquired pneumonia, 50 000 cases of bacteraemia, 3 000 cases of meningitis, and up to 6 million cases of otitis media (Tomasz, 1997). Pneumococcal infection is most frequent at the extremes of age, in infants up to two years and in adults over 65 years. This is illustrated in a study from Temuco, Chile (Inostroza et al., 2001) in which the incidence of invasive pneumococcal disease was 348, 54 and 234 per 100 000 persons in infants (<2 years), children/adults (5-65 years) and the elderly (>65 years) respectively.

Grouping	Disorder	Site of infection
Superficial infections		
	Conjunctivitis	Eye
	Otitis media	Middle ear
Deep infections		
	Pneumonia	Lung
	Bacteraemia	Blood stream
	Meningitis	CSF/Brain
Atypical infections		
	Endocarditis	Heart valve
	Necrotizing Fasciitis	Skin, deep tissue

Table 1.1 Sites of potential pneumococcal infection

S. pneumoniae is a component of the normal bacterial flora of the nasopharyx. In the absence of injury to the respiratory tract pneumococci exist in a commensal state with humans, however the pneumococcus has the potential to infect and cause disease in a large number of niches in the

human body (Table 1.1). This ability has lead to a huge array of infections that may be attributed to the pneumococcus (Taylor and Sanders, 1999). Factors which predispose individuals to pneumococcal infection in an adult population include; alcoholism, HIV infection, splenectomy, multiple myeloma, connective tissue disease, steroid use, diabetes mellitus, and intravenous drug use (Taylor and Sanders, 1999).

Disruption of the cells lining the respiratory tract, by viral, physical, or chemical challenge predisposes the host to bacterial infection. Such disruption causes congestion throughout the respiratory tract, including the nose, nasopharynx, eustachian tube and middle ear. Congestion of the eustachian tube results in obstruction of its narrowest portion, the isthmus. This prevents drainage of secretions from the middle ear, which accumulate. If pathogenic bacteria from the nasopharynx are present in the middle ear after such an obstruction, otitis media – infection of the middle ear – will result (Klein and Bluestone, 1998). In most instances when an etiological agent is isolated, *S. pneumoniae* is the organism most frequently recovered, or is second only to *H. influenzae* (Klein and Bluestone, 1998).

Disturbance of the lower respiratory tract of healthy individuals may be followed by infection of the lungs. Bacteria reaching the alveoli of the lungs are usually rapidly cleared. Injury of the alveolar lining cells by viral (e.g., influenza) infection or other means will delay bacteria clearance (Hament et al., 1999). The prolonged presence of bacteria allows the establishment of infection and results in pneumococcal pneumonia. This may be complicated further by the presence of alcohol, anaesthetics or corticosteroids, which retard the movement of polymorphonuclear leukocytes, and thus facilitates the spread of pneumonia.

If the host immune system fails to contain the infection, bacteria may extend from the lungs to the hilar lymph node. From the lymph node bacteria may gain access to the systemic circulatory system via the thoracic duct, resulting in pneumococcal bacteraemia. Once bacteraemia is established, the invading pneumococcus is capable of evading host immune systems by virtue of its polysaccharide capsule (Tunkel and Scheld, 1993). Meningitis may follow if the bacteria traverses the blood-brain barrier, and establishes a focus of infection. The cerebral spinal fluid (CSF) provides the infecting organism with a sanctuary, devoid of many of the host's immune defences, in which the invading bacteria may replicate rapidly (Tunkel and Scheld, 1993, Feigin and Pearlman, 1998). Inflammation of the meninges occurs as a result of the presence of bacteria and their associated products in the CSF. Pneumococcal lysis, whether autolysis by pneumolysin or antibiotic mediated, directly attracts and activates phagocytes. The resulting

inflammation is thought to be responsible for the morbidity and mortality associated with pneuomococcal meningitis (Alonso De Velasco et al., 1995).

1.3.2 Pneumococcal pathogenicity

Pneumococcal pathogenicity has been attributed to a number of structures, mostly associated with the surface of the cell. Although a great number of virulence factors have been proposed (see Alonso De Velasco and Jedrzejas for reviews (Alonso De Velasco et al., 1995, Jedrzejas, 2001)) it is likely there are more yet to be identified. The following sections relate to two of the best described of the pneumococcal virulence factors; the polysaccharide capsule, and the cytotoxin pneumolysin.

1.3.2.1 The pneumococcal capsule

The pneumococcus is enveloped by a polysaccharide capsule that forms the outermost layer of all fresh clinical isolates. The pneumococcal capsule is considered the primary virulence factor based on the observation that loss of the capsule renders an isolate significantly less efficient at causing disease than the encapsulated parent from which it was derived (Griffith, 1928, Watson and Musher, 1990). Although an important virulence factor, the precise role played by the capsule during infection is not completely understood. It is well documented that some capsular types are more adept at causing invasive disease than others. For example, of 1477 isolates serotyped in Brazil over a 16-year period, 51.9% of the isolates belonged to eight serotypes (1, 6B, 14, 6A, 18C, 3, 5, 23F) of the 64 different capsular types identified (Brandileone et al., 1995). The differential association of serotypes causing invasive disease seems to be a function of the chemical composition of the capsule (Alonso De Velasco et al., 1995). The capsule has been shown to be required for colonization of the nasopharynx of mice, which is necessary prerequisite for pathogenesis (Magee and Yother, 2001). The major mechanism of virulence attributed to the pneumococcal capsule is its resistance to phagocytosis and lack of activation of the alternative complement pathway (Alonso De Velasco et al., 1995).

1.3.2.2 Pneumolysin

Pneumolysin is a 53 kDa cytotoxic protein that belongs to the family of thiol-activated toxins, and shares sequence identity with streptolysin O (*S. pyogenes*) and lysteriolysin O (*Listeria monocytogenes*) (Billington et al., 2000). Unlike cytotoxins from other bacterial species, pneumolysin is cytoplasmic and is released from the pneumococcal cell upon lysis (Jedrzejas, 2001). Pneumolysin has at least two biological activities that contribute to virulence in animal models; cytolytic activity and the ability to activate complement (Mitchell et al., 1991,

Jedrzejas, 2001). The cytotoxic nature of pneumolysin is due to the toxin binding cholesterol in host cell membranes, with subsequent disruption of membranes by forming large oligomeric pores (Rossjohn et al., 1998). This activity inhibits the action of ciliated mucosal cells, and aids colonisation by preventing the host from clearing bacteria. Pneumolysin has also been shown to activate the complement pathway (Mitchell et al., 1991). *In vivo*, this activity would stimulate an inflammatory response, a characteristic and key component of pneumococcal infection. Evidence for pneumolysin as an important virulence factor is supported by the fact that strains with a deleted pneumolysin have greatly attenuated virulence in mouse models (Berry and Paton, 2000).

1.4 Treatment of Pneumococcal Infections

Historically, the management of pneumococcal infections can be divided into three phases; the pre-antibiotic era and immunotherapy, the clinical introduction of antibiotics, and the current phase of increasing resistance to antibiotics.

1.4.1 Pre-penicillin treatment

Shortly after the elucidation of the pneumococcus as a major cause of lobar pneumonia, there was a period of active interest in the immunology of the organism. It was soon noted that animals could be immunised with cell-free pneumococcal filtrates, and this would protect the animal against subsequent pneumococcal infection. Furthermore, serum taken from an immunised animal could be used to protect naïve animals from experimental pneumococcal infection (Austrian, 1981). This observation spawned the first and only successful treatment option (immunotherapy) for pneumococcal infection until the advent of antibiotic chemotherapy. Immunotherapy was initially hampered by the fact that different capsular types had not yet been distinguished. However, by 1910 different capsular types had been recognised, and four groups described; types I, II, III and IV (type IV essentially being everything which did not fall into the first three). Subsequently, using antisera to treat pneumococcal pneumonia proved to be quite effective (Dubos, 1976).

Immunotherapy has two major drawbacks. Firstly, immunotherapy is dependant on the serotype of the infecting organism and subsequent administration of the corresponding antisera as early as possible. This process is time consuming, and the efficacy of the antisera diminishes with delayed treatment. Secondly, treatment with antisera provides no advantage for localised infections such as meningitis. Consequently the focus management of pneumococcal infections shifted from the treatment of the disease, to prevention. Research into the use of pneumococcal

vaccines coincided with the use of antisera for treatment, however the efficacy of vaccines was more difficult to judge. The most persuasive report showing the effectiveness of a vaccine was published in 1945 on recruits at a US air force base (MacLeod et al., 1945). MacLeod and colleagues administered a pneumococcal vaccine, containing capsular polysaccharides from types 1, 2, 5 and 7, to a group of 8586 men. Over the course of the study, only four cases of pneumonia caused by a type included in the vaccine was observed in the treated population, compared to 26 cases in the control population (8449 men). Pneumonia caused by types not included in the vaccine was similar for both immunized and non-immunized groups (56 and 59 cases respectively).

Although by 1945 considerable progress had been made in the formulations of vaccines for preventing pneumococcal disease, this also coincided with the introduction of antibiotics. Antibiotics have the advantage of being effective against all isolates of pneumococci, regardless of serotype. They are also able to clear localised infection, which were previously non-treatable. Interest in pneumococcal immunology subsequently diminished rapidly, and with the exception of some serotype surveillance, such research was effectively halted.

1.4.2 The antibiotic era

In 1928 Alexander Fleming described the antibacterial properties of a compound produced by a member of the *Penicillium* genus of fungus (Fleming, 1929). The compound, dubbed penicillin, inhibited a variety of microorganisms, particularly Staphylococci and Streptococci; *"It is on the pyogenic cocci and on bacilli of the diptheria group that the action is most manifest"*. Chain and co-workers (1940) followed up the work of Fleming and investigated the potential of penicillin as a chemotherapeutic agent. The experiments described provide the first evidence of penicillin's efficacy in treating bacterial infections. Penicillin was shown to be effective in the treatment and clearance of three pathogenic bacterial species, including *S. pyogenes*, in a mouse model. Thus was borne the antibiotic era. To this day β-lactams, including penicillins and cephalosporins, have remained the most effective antibiotics in the treatment of streptococcal disease.

The introduction of antibiotics greatly reduced the mortality associated with pneumococcal infection. Prior to antibiotic chemotherapy, the mortality associated with pneumococcal pneumonia, bacteraemia and meningitis was 20%, 50% and 80-100% respectively. Appropriate antibiotic treatment, predominantly with penicillin, has reduced these statistics to 5%, 20% and 30% respectively (Tomasz, 1997). Although penicillin, due to its low toxicity, low cost and high

efficacy, is considered the antibiotic of choice for anti-pneumococcal chemotherapy, many other agents are also highly effective. In some instances (e.g., allergy to penicillin), treatment with penicillin may not be an option. In such cases antibiotics such as erythromycin, tetracycline, co-trimoxazole and chloramphenicol are suitable alternatives for treatment.

1.4.3 Post-penicillin era

1.4.3.1 Antibiotic resistance and new antibiotics

In recent years, the spectre of increasing antibiotic resistance in many bacterial pathogens, including the pneumococcus, has become a serious therapeutic concern (Neu, 1992). Consequently, much research has been conducted in formulating new anti-pneumococcal treatment regimes in a bid to curb rising antibiotic resistance in the pneumococcus (Appelbaum, 1995). The unmet need for new antibiotic agents has prompted pharmaceutical companies to attempt to uncover novel agents effective in treating bacterial disease caused by resistant organisms. Data generated from microbial genomics is being exploited in a bid to identify new cellular targets, in order to bypass existing resistance mechanisms. To this end, the US pharmaceutical industry spent $2.8 billion on research of infectious disease in 1997 (Bax et al., 2000).

Although not a new class of antibiotic, fluoroquinolones, particularly the more modern members (e.g., moxifloxacin) have gained popularity in the treatment of pneumococcal infections. Other options for anti-pneumococcal chemotherapy include novel antimicrobial compounds such as the ketolides (telithromycin), quinupristin/dalfopristin (Synercid) and oxazolidinones (linezolid). Comparative activities of some of these agents are listed in Table 1.2 (adapted from Ball (Ball, 1999)).

The fluoroquinolone antibiotic, ciprofloxacin, was shown to have fairly good clinical efficacy in the treatment of pneumonia. Krumpe and colleagues (1999) showed ciprofloxacin mono-therapy had a clinical success rate (defined as dissipation of symptoms) of 85%, compared to 83% for standard therapy (β-lactam) when treating pneumonia. However, ciprofloxacin appeared less effective than standard therapy for bacteriological eradication (82% and 100%, respectively). Furthermore, in vitro susceptibility data on the susceptibility of pneumococci to fluoroquinolones have shown older agents such as ciprofloxacin have MICs that tend to cluster around their respective breakpoints (2.0 μg/ml) (Visalli et al., 1996, Ednie et al., 1998, Hoellman et al., 1999). The introduction of newer fluoroquinolones such as levofloxacin, trovafloxacin and moxifloxacin, which have greater activity against Gram-positive organisms,

has renewed interest in using these agents for treating *S. pneumoniae* infections (Appelbaum and Hunter, 2000).

Ketolides are novel, 14-membered-ring, semi-synthetic macrolides (Hunter, 1998). They are active against *S. pneumoniae* including isolates that are resistant to conventional macrolide antibiotics (Chu, 1999). Morosini and colleagues (2001) showed the activity of one such ketolide, telithromycin, against erythromycin-resistant pneumococci. Telithromycin was active against 98.9% (MICs ≤ 0.5 μg/ml) of 203 Spanish pneumococcal isolates, while erythromycin was active against 61.0% (MICs ≤ 0.25 μg/ml). MIC_{90} values were 0.06 μg/ml and >64 μg/ml for telithromycin and erythromycin respectively. Furthermore, in a Canadian study (Hoban et al., 2001) of 215 macrolide-resistance pneumococcal isolates, the MIC_{90} of two ketolides, telithromycin and ABT-773 were determined to be 0.06 and 0.03 μg/ml respectively, regardless of the macrolide-resistance genotype. Ketolides may well be a useful alternative therapy for pneumococci resistant to other macrolides.

Antibiotic	MIC_{90}		
	Penicillin-sensitive (<0.12 μg/ml)	Penicillin intermediate (0.12-1.0 μg/ml)	Penicillin-resistant (>1.0 μg/ml)
β-lactams			
Penicillin	0.06	1.0	4
Ceftriaxone	0.03	0.5-1.0	1-2
Vancomycin	0.25	0.5	0.5-1.0
Fluoroquinolones			
Trovafloxacin	0.12	0.12	0.06
Moxifloxacin	0.12	0.12	0.12
Sparfloxacin	0.5	0.5	0.5
Levofloxacin	1	1	1
Ciprofloxacin	1	1	1-2
Other Novel Agents			
Ketolides	0.12	0.12	0.12
Quinupristin/dalfopristin	1	1	1
Oxazolidinones	0.5-1.0	0.5-1.0	0.5-1.0

Table 1.2 Comparative activities of antibiotic agents on penicillin-susceptible, intermediate, and resistant pneumococci.

Quinupristin/dalfopristin is a two component antibiotic consisting of quinupristin (streptogramin B) and dalfopristin (streptogramin A) in the ratio of 30:70 (w/w) (Nadler et al., 1999). The two components have a synergistic mode of action, both targeting the bacterial ribosome. The drug is currently reserved for treatment of serious infections by Gram-positive organisms including isolates that currently available drugs are ineffective. The quinupristin/dalfopristin MIC_{50} and MIC_{90} for *S. pneumoniae* is typically 0.25 μg/ml and 0.5 μg/ml respectively, regardless of the level of penicillin resistance (Doern et al., 2001).

Linezolid is a member of a new class of synthetic antibiotics, named the oxazolidinones, unrelated to any other currently available agent. Linezolid binds to the 50S ribosomal subunit near the interface with the 30S subunit, which results in the inhibition of the initiation of protein synthesis (Corti et al., 2000). Linezolid is active against Gram-positive cocci, including multi-resistant isolates. A Taiwanese study found linezolid to have a MIC range of 0.125-0.5 μg/ml when tested against 54 pneumococcal isolates (Fang et al., 2001). No cross-resistance to other antimicrobials was noted.

1.4.3.2 Pneumococcal vaccine

The recent emergence of antibiotic resistance in *S. pneumoniae* has emphasized the importance of prevention and control of pneumococcal disease, most practically achieved by vaccination. Unfortunately, complacency derived from the introduction of antibiotic chemotherapy for treating pneumococcal infection has significantly set back vaccine development for this organism. In 1946 commercial hexavalent pneumococcal vaccines were produced, but were subsequently withdrawn because of the wide availability and efficacy of penicillin (Lee et al., 1991). However, Austrian and Goldman (1964) found that antibiotic therapy had little effect on the mortality of pneumococcal pneumonia within the first five days of treatment (Austrian and Gold, 1964). This was a startling insight, as at the time many were of *"...the impression that pneumococcal disease no longer constitutes a serious medical problem"* (Austrian and Gold, 1964).

Renewed interest in vaccination against pneumococcal infections saw FDA approval of a 14-valent vaccine in 1977, which was followed by a 23-valent vaccine six years later (Lee et al., 1991). The 23-valent vaccine contains purified capsular polysaccharides of types 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Efficacy of the 14-valent and 23-valent vaccines for preventing invasive disease due to serotypes covered by the vaccine were 53% and 60%, respectively (Butler et al., 1993).

Unfortunately the currently available 23-valent pneumococcal vaccine does not provide adequate protection to children less than two years old, who are at greatest risk of disease (Klein, 1999). Subsequently alternative vaccine candidates are being pursued. A seven-valent pneumococcal vaccine has recently been licensed for use in the United States (Mulholland, 2000). It is hoped this may reach the success achieved with the *Haemophilus influenza* type b vaccine, introduced in 1990.

1.5 Antibiotic Resistance in *S. pneumoniae*

1.5.1 Development and spread of pneumococcal resistance

S. pneumoniae was thought to be consistently sensitive to penicillin (MIC 0.006 μg/ml), and for at least two decades this was the case. Possibly the first documented account of penicillin resistance in the pneumococcus occurred in 1965. Investigators in Boston noted that among 200 clinical isolates of *S. pneumoniae*, two isolates had MICs in the 0.1-0.2 μg/ml range (Kislak et al., 1965). However, these investigators failed to recognise the significance of their observations. It was not until 1967 that Hansman & co-workers identified a resistant pneumococcus in Sydney, Australia (Hansman and Bullen, 1967). In this instance the resistance strain had a penicillin MIC of 0.6 μg/ml.

Following these initial observations, sporadic descriptions of penicillin-resistant pneumococci began to surface in the literature. Fifteen serotype 4 penicillin insensitive (MIC 0.5 μg/ml) pneumococci were recovered from a village in New Guinea (Hansman et al., 1971a, Hansman et al., 1971b). The first clinical description of a resistant pneumococcus from North America was an isolate recovered from a 68-year-old man in Alberta, Canada (Dixon, 1974). The isolate was serotype 9 and had a penicillin MIC of 0.32 μg/ml. In the same year, a report of relapsing pneumococcal meningitis was described in Chicago (Naraqi et al., 1974). In this instance a serotype 23 pneumococcus was recovered with a penicillin MIC of 0.25 μg/ml. This case was also notable in that it pointed out pneumococcal infections in difficult to treat sites (e.g., CSF), may require higher than recommended doses of penicillin to successfully eradicate infection. The first isolation of a penicillin-resistant pneumococcus in Britain was recovered from a 10-month-old girl presenting with meningitis (Howes and Mitchell, 1976). The isolate was serotype 14 and had a penicillin MIC of 0.37 μg/ml.

Around the same time pneumococcal penicillin resistance was noted, resistance to non-β-lactam antibiotics was also being described. To many at the time, the increased resistance to non-β-

lactam antibiotics was more concerning than those of penicillin itself, as the increases were more dramatic and absolute (Gunnison et al., 1968, Finland, 1971). While increased penicillin resistance was certainly significant when compared to sensitive isolates, the MICs were still well below the therapeutic levels of antibiotic that could be achieved in serum. Furthermore, the initial descriptions of penicillin resistance were deemed too geographically isolated to be considered significant, as cited in a report by Finland (1971);

> "It [the 1971 Hansman *et al* report] raises the spectre of a new and increasing problem of resistance to penicillin in the treatment of pneumonia and other serious pneumococcal infections. Although such a possibility cannot be completely dismissed, the particular epidemiologic circumstances in the report from New Guinea, and the special properties of the strain of pneumococcus involved, make such a prospect extremely remote"

The same report goes on to dismiss the potential need for diagnostic screening of pneumococcal resistance to penicillin, but does suggest a need to investigate resistance to other classes of antibiotics. Consequently, a brief review of pneumococcal resistance to several other clinically useful antibiotics, in particular; tetracycline, erythromycin, co-trimoxazole and chloramphenicol, is included here.

Tetracycline-resistance was first noted in Australia by Evans and Hansman (1963). A resistant isolate was recovered from a 10-month infant with meningitis. The tetracycline MIC of this strain was determined to be 11 μg/ml. The isolate was susceptible to other non-related antimicrobial compounds, and was eventually cleared with penicillin. Over the following two years, an additional 37 tetracycline-resistant isolates were recovered in an Australian hospital, with MICs ranging from 15-100 μg/ml (Hansman and Andrews, 1967). Within two weeks of the initial observation, Richards and Rycroft (1963) reported on the culture of four similar isolates from St. Pacras hospital in London. Of these four strains, three belonged to serogroup 9 and the other to serotype 11. In all four cases patients had been treated with tetracycline shortly (one week - two months) before the recovery of the resistant organism. Later the same year in a Liverpool hospital, tetracycline-resistant pneumococci were isolated from ten patients (Turner, 1963). In this instance all ten organisms were capsular type 7, suggesting cross-infection may have occurred within the hospital. Of the ten patients, five had received tetracycline a minimum of 12 days before the isolation of the resistant pneumococcus.

The macrolide antibiotic, erythromycin, is considered second only to penicillin for treating pneumococcal infections. Resistance to erythromycin, as well as lincomycin, was first reported

in 1967 (Kislak, 1967). A type 6 pneumococcus was isolated from a 10-year-old boy in New York. The patient had been treated within the two months previous with both erythromycin and lincomycin. A further report from Australia of a serotype 11, erythromycin-resistant pneumococcus appeared later that year (Cooper et al., 1968).

Cotrimoxazole is a combination of two agents; trimethoprim and sulfamethoxazole. Trimethoprim inhibits dihydrofolate reductase (DHFR), and subsequently blocks the reduction of dihydrofolate to tetrahydrofolate. This deprives the cell of a vital precursor for the synthesis of purines, thymidylate and various amino acids and vitamins. The first pneumococcal isolate with resistance to co-trimoxazole was described in 1972 (Howe and Wilson, 1972). In this instance a 58-year-old woman was admitted to hospital with acute exacerbation of chronic bronchitis. Culture of a sputum specimen recovered a serogroup 23 pneumococcus resistant to co-trimoxazole, but sensitive to non-related antibiotics. The patient had received a course of co-trimoxazole one month previously.

Chloramphenicol is a broad-spectrum antibiotic which owes its activity to its strong inhibitory effect upon the peptidyl transferase activity of the 50S ribosomal subunit. Chloramphenicol is a bacteriostatic agent, but may exert a bactericidal effect at two to four times the MIC towards Gram positive cocci. Pneumococci resistant to chloramphenicol were first described by Cybulska in Poland (Cybulska et al., 1970). A survey of pneumococcal carriage in Nigeria in 1977 found 14% of isolates resistant to chloramphenicol (Hansman, 1978).

In 1977 the first report of multi-drug resistant pneumococci emerged from Durban, South Africa. Resistant pneumococci were isolated from five patients with systemic infection, including three cases of meningitis. These isolates were highly resistant to penicillin (MIC 4-8 μg/ml) and also exhibited resistance to chloramphenicol and streptomycin (Appelbaum et al., 1977). All five isolates were capsular type 19A. At a similar time, 300 miles away in Johannesburg, similar resistant pneumococci were isolated. The first case was from a three year old boy who developed pneumonia post-operatively. A serotype 19A pneumococcus was recovered. The isolate was resistant to penicillin, erythromycin, tetracycline, chloramphenicol, co-trimoxazole and various aminoglycosides (Anonymous, 1977). Following this index case, potential carriers of the resistant pneumococcus were surveyed at two Johannesburg hospitals (Jacobs et al., 1978). Cultures from 427 patients and 363 staff members of Baragwanath hospital were performed yielding penicillin-resistant pneumococci in 83 patients (19%) and eight staff members (2%). Of these, 51 were multi-drug resistant 19A, and the remaining 32 were serotype

6A or 19A, and were resistant to penicillin, tetracycline and chloramphenicol either singly or in combination. At the second hospital a type 19A multi-resistant pneumococcus was isolated from 77 patients.

Thus began the South African epidemic of penicillin-resistant pneumococci – an ongoing problem in this country today (Koornhof et al., 1992, Klugman and Saunders, 1993). Unlike previous sporadic reports of resistance, epidemiological links could be made, and it appeared as if the epidemic was being caused by the dissemination and spread of distinct pneumococcal lineages. Surveillance of penicillin-resistant pneumococci in South Africa between 1979 and 1986 showed an increase in prevalence among isolates from invasive disease from 4.9% in 1979 to 14.1% in 1986 (Klugman and Koornhof, 1988). The most frequently recovered serogroups during this period were; 6 (59%), 19 (25.9%), and 14 (12.7%). Throughout the 1970s-80s, reports of pneumococcal resistance were published from all parts of the world. These have been summarised in several reviews (Appelbaum, 1987, Klugman, 1990, Appelbaum, 1992). A summary of the worldwide distribution of resistant pneumococci is shown in Figure 1.1(a) (adapted from Appelbaum (Appelbaum, 1992)). This figure illustrates prevalence data that was accurate until around 1990. A more current approximation of worldwide prevalence is shown for comparison in Figure 1.1(b), based on the data in Table 1.3.

Location	Years	# Pen res. (%)	Source	Reference
Africa				
Ivory Coast	1996-97	31/138 (22.5)	Clinical	(Benbachir et al., 2001)
Kenya	1991-92	58/223 (26)	Clinical	(Kell et al., 1993)
Morocco	1996-97	9/98 (9.2)	Clinical	(Benbachir et al., 2001)
Senegal	1996-97	50/81 (61.7)	Clinical	(Benbachir et al., 2001)
South Africa	1997	20/66 (30.3)	Clinical	(Felmingham and Gruneberg, 2000)
Tunisia	1996-97	24/58 (41.4)	Clinical	(Benbachir et al., 2001)
Zambia	1994	7/126 (5.5)	Carriage	(Woolfson et al., 1997)
Asia				
Bangladesh	1993-97	46/362 (12.7)	Clinical	(Saha et al., 1999)
China	1996-97	5/51 (9.8)	Clinical	(Song et al., 1999)
Hong Kong	1997	33/54 (61.1)	Clinical	(Felmingham and Gruneberg, 2000)
India	1996-97	7/183 (3.8)	Clinical	(Song et al., 1999)
Indonesia	1996-97	7/33 (21)	Clinical	(Song et al., 1999)
Japan	1996-97	55/84 (65.3)	Clinical	(Song et al., 1999)
Korea	1996-97	141/177 (79.7)	Clinical	(Song et al., 1999)
Malaysia	1996-97	3/34 (9.0)	Clinical	(Song et al., 1999)
	1995-96	19/273 (7.0)	Clinical	(Rohani et al., 1999)
Pakistan	1993-94	118/229 (51.5)	Clinical	(Qazi, 1999)
Saudi Arabia	1997	15/31 (48.4)	Clinical	(Felmingham and Gruneberg, 2000)
Singapore	1997-99	114/180 (63)	Clinical/Carriage	(Soh et al., 2000)
	1996-97	19/84 (23.1)	Clinical	(Song et al., 1999)
Sri Lanka	1996-97	17/41 (41.2)	Clinical	(Song et al., 1999)
Taiwan	1996-97	120/200 (60)	Clinical	(Hsueh et al., 1999)
	1996-97	310/550 (56.4)	Clinical	(Fung et al., 2000)
Thailand	1996-97	73/126 (57.9)	Clinical	(Song et al., 1999)
Vietnam	1996-97	28/46 (60.8)	Clinical	(Song et al., 1999)
	?-1997	212/399 (51)	Carriage	(Parry et al., 2000)
Oceania				
Australia	1997	259/1020 (25.4)	Clinical	(Turnidge et al., 1999)
New Zealand	1999-97	2085/10976 (19)	Clinical	(Brett and Ellis-Pegler, 2001)
	2000	1498/5830 (25.7)	Clinical	(Anonymous, 2001)
Europe				
Austria	1994-96	68/1385 (4.9)	Clinical	(Georgopoulos et al., 1998)
Belgium	1994-98	542/5486 (9.9)	Clinical	(Verhaegen et al., 2000)
Czech Republic	1997	5/93 (5.4)	Clinical	(Felmingham and Gruneberg, 2000)
France	1993-94	207/363 (57)	Clinical	(Moissenet et al., 1997)
	1997	90/181 (49.7)	Clinical	(Felmingham and Gruneberg, 2000)
Germany	1998-99	62/961 (6.6)	Clinical	(Reinert et al., 2001)
Greece	1997	15/132 (11.4)	Carriers	(Tsolia et al., 1999)
Hungary	1996	46/127 (36.2)	Clinical	(Felmingham and Gruneberg, 2000)
Italy	1997	17/121 (14)	Carriers	(Ronchetti et al., 1999)

Location	Years	# Pen res. (%)	Source	Reference
Netherlands	1996	3/65 (4.6)	Clinical	(Felmingham and Gruneberg, 2000)
Poland	1997	(14.4)	Clinical	(Overweg et al., 1999)
Portugal	1996	66/277 (24)	Carriers	(De Lencastre et al., 1999)
Rep. Ireland	1997	21/87 (24.1)	Clinical	(Felmingham and Gruneberg, 2000)
Scotland	1993-95	1/98 (1)	Clinical	(McKenzie et al., 2000)
Slovak Republic	1997	16/60 (26.7)	Clinical	(Felmingham and Gruneberg, 2000)
Spain	1999-2000	197/300 (65.6)	Clinical	(Oteo et al., 2001)
	1996-97	669/1113 (60.1)	Clinical	(Baquero et al., 1999)
	1990-96	4527/9243 (49)	Clinical/Carriage	(Fenoll et al., 1998)
Sweden	1993-97	694/10735 (6.5)	Carriers	(Kamme et al., 1999)
Switzerland	1993-94	25/351 (7.1)	Clinical	(Wust et al., 1995)
UK	1997	14/111 (12.6)	Clinical	(Felmingham and Gruneberg, 2000)
North America				
Alaska	1991-98	109/1037 (10.5)	Clinical	(Rudolph et al., 2000)
Canada	1991-98	104/1538 (6.7)	Clinical	(Greenberg et al., 2002)
US	1999-2000	524/1531 (34.2)	Clinical	(Doern et al., 2001)
	1998-99	632/4296 (14.7)	Clinical	(Sahm et al., 2001)
	1997-98	472/1601 (29.5)	Clinical	(Doern et al., 1999)
Mexico	1997	31/63 (49.2)	Clinical	(Felmingham and Gruneberg, 2000)
	1993-95	106/220 (48.2)	Clinical	(Echaniz-Aviles et al., 1998)
South America				
Argentina	1993-96	123/505 (24.4)	Clinical	(Rossi et al., 1998)
Brazil	1993-96	190/1525 (15.1)	Clinical	(Brandileone et al., 1998)
Chile	1997	31/99 (31.3)	Clinical	(Jacobs and Appelbaum, 2000)
Columbia	1994-96	64/409 (15.6)	Clinical	(Castaneda et al., 1998)
Panama	1997	14/61 (23.0)	Clinical	(Jacobs and Appelbaum, 2000)
Venezuela	1997	16/73 (21.9)	Clinical	(Jacobs and Appelbaum, 2000)
West Indies	1997	6/84 (7.1)	Clinical	(Jacobs and Appelbaum, 2000)

Table 1.3 Worldwide prevalence of penicillin-resistant isolates of *S. pneumoniae*.
The proportion of resistant isolates includes all isolates of intermediate resistance (≥ 0.12 μg/ml). Preference was given to reports presenting clinically relevant data, rather than those of asymptomatic carriage.

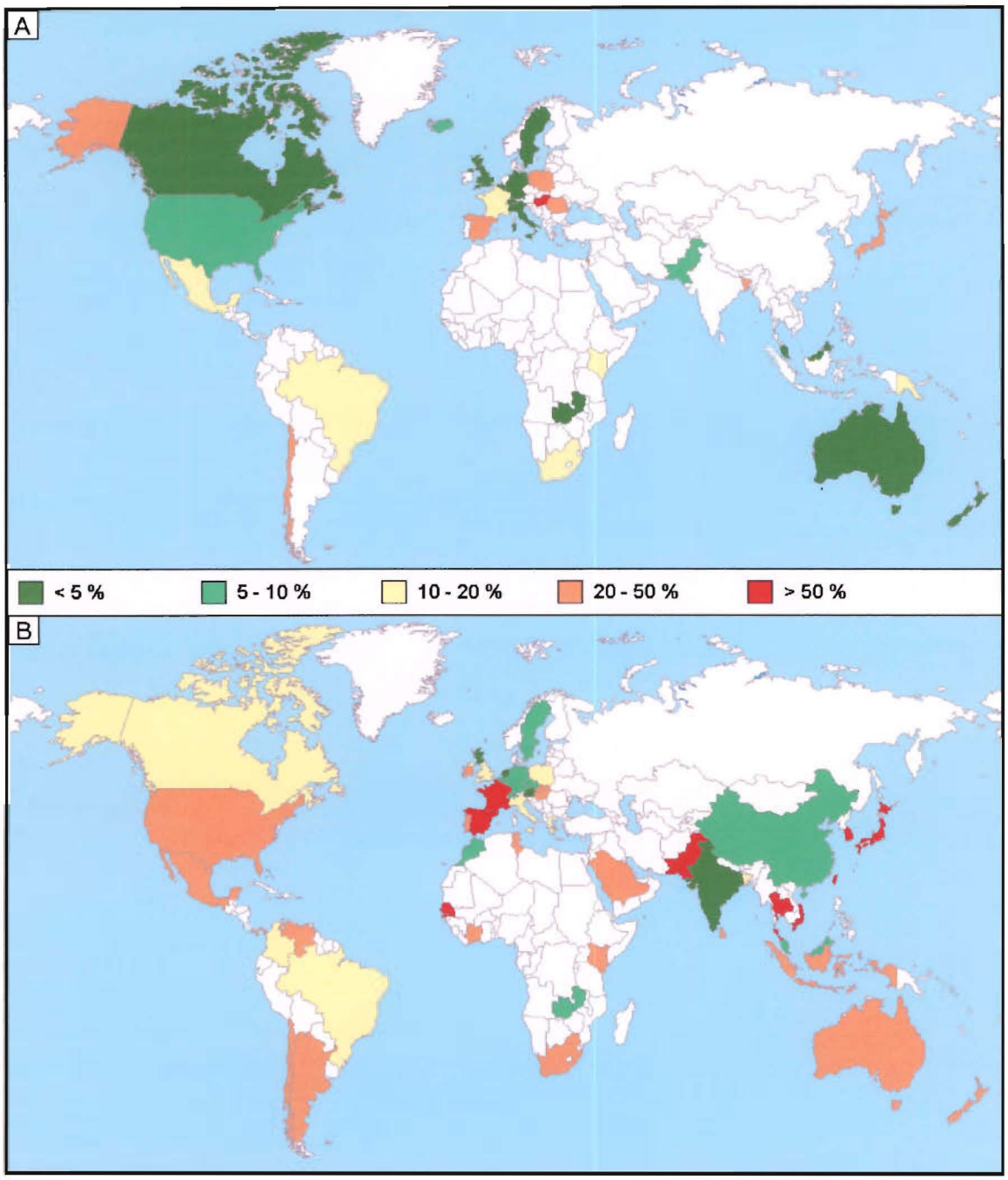

Figure 1.1 Worldwide prevalence of penicillin non-susceptible pneumococci.
Based on; (a) global prevalence data, accurate until around 1990 (adapted from Appelbaum (Appelbaum, 1992)); (b) a current approximation of worldwide prevalence of non-susceptible pneumococci (based on the data in table 1.3). Unshaded regions represent areas where current prevalence data was unavailable.

1.5.1.1 Nomenclature of penicillin resistance

It is worth a brief mention of the nomenclature involved in pneumococcal penicillin resistance. The pneumococcus acquires penicillin resistance in a stepwise fashion, rather than obtaining absolute resistance imparted by a β-lactamase. Consequently, there has been a potential need to

distinguish between intermediate resistance (MIC 0.1–1.0 μg/ml) and full resistance (MIC ≥2.0 μg/ml). This distinction is particularly relevant for the management of pneumococcal meningitis, which may be complicated by moderate increases in penicillin resistance. This has led to a veritable plethora of acronyms having been introduced in the literature to describe resistant pneumococci, some of which include; PN(S)SP (penicillin non-susceptible *Streptococcus pneumoniae*; (McEllistrem et al., 2000a, Greenberg et al., 2002)), PRSP (penicillin-resistant *Streptococcus pneumoniae*; (Bryan, 1999)), (M)DRSP (multi-drug resistant *Streptococcus pneumoniae*; (Ball, 1999, Greenberg et al., 2002) and RRP (relatively resistant pneumococcus; (Saah et al., 1980)). For a more exhaustive list of related terms, see the 1990 review of Klugman (Klugman, 1990).

For the purposes of the current text, many of the above terms have been employed, although the use of the corresponding acronyms has been avoided to minimise confusion. Unless otherwise stated, resistance refers to isolates with a penicillin MIC of ≥0.1 μg/ml (i.e. both intermediate and highly resistant organisms). Wherever possible throughout this text, it has been endeavoured to also include actual MICs to further avoid confusion.

1.5.2 Penicillin resistance in *S. pneumoniae*

In many bacteria, a β-lactamase enzyme mediates resistance to β-lactams. Such enzymes cleave the β-lactam ring of penicillins rendering them inactive (Ghuysen, 1991, Bush and Miller, 1998). The pneumococcus differs from most bacteria in this regard as a β-lactamase has never been detected (Robins-Brown et al., 1979). Resistance has instead been identified to be due to changes in the affinity of the target molecules, the penicillin-binding proteins (PBPs), toward penicillin. This section serves as a background review of the function of PBPs and their role in peptidoglycan biosynthesis (for reviews see (Blumberg and Strominger, 1974, Georgopapadakou, 1993, Goffin and Ghuysen, 1998)). The penicillin resistance mechanism will be covered in more detail in Chapter 5.

1.5.2.1 Biological function of PBPs

PBPs are enzymes that selectively bind and are inhibited by penicillin. They are membrane bound enzymes, and involved in cell wall synthesis and maintenance. PBPs are readily visualised after incubating cell wall extracts in the presence of radioactively labelled penicillin after separation by SDS-PAGE; the binding of penicillin to the enzymes facilitated their discovery. PBPs are a group of enzymes common to all bacteria, with the exception of non-peptidoglycan containing bacteria (e.g., mycoplasmas).

Penicillin binding proteins themselves are classified as high or low molecular weight proteins. The high molecular weight PBPs are multimodular; that is they have at least two catalytic functions encoded in the same polypeptide; the low molecular weight PBPs are monofunctional. *S. pneumoniae* contains six PBPs; five high-molecular weight (PBPs 1a, 1b, 2a, 2b and 2x) and one low molecular weight (PBP 3). The high molecular weight PBPs are further subdivided into classes. The differentiation into classes is based upon comparison of amino acid sequences (Goffin and Ghuysen, 1998). The pneumococcal PBPs 1a, 1b and 2a belong to class A and PBPs 2b and 2x belong to class B.

All PBPs possess a common penicillin-binding domain, which is responsible for the enzymatic inactivation of these enzymes by the covalent binding of the drug to the active site serine residue. In the high-molecular-weight PBPs this penicillin-binding domain is the C-terminal portion of the enzyme. Structurally, PBPs contain a transmembrane "anchor" fused to the amino end of a transglycosylase (non-penicillin binding) module, which is in turn fused to the amino end of a D, D-transpeptidase (penicillin binding) module. The transpeptidase module contains conserved amino acid motifs of SXXK, SXN and KT(S)G. The first of these motifs contains the active-site serine residue, the target of β-lactam antibiotics.

1.5.2.2 Biological function of peptidoglycan

The cell envelope is an indispensable structural feature of many prokaryotes. It is responsible for providing cellular support and maintenance of morphology (Koch, 2000a, Koch, 2000b). Bacteria lacking cell walls are typically not free living, and must be maintained in an environment where the external osmotic pressure equals the cell internal osmotic pressure. The cell walls of all bacterial species have common aspects (Schleifer and Kandler, 1972). Both Gram-positive and Gram-negative bacteria have a membrane that surrounds the cytoplasm. The peptidoglycan is located external to this membrane. Gram-negative bacteria also have a second, outer membrane (Figure 1.2). In each case, the peptidoglycan layer provides the cell structural stability and shape.

Peptidoglycan is a continuous, covalently linked network of muropeptides that surround the cell. Peptidoglycan is structurally similar amongst all eubacterial species. It consists of glycan strands, made up of alternating β-1,4-linked units of *N*-acetylglucosamine and *N*-acetylmuramic acid, which are cross-linked by short peptides (Figure 1.3). These cross-linking peptides are typically 4-5 residues in length, with an alternating series of L- and D- amino acids. The peptide units are linked to the carboxyl group of the *N*-acetylmuramic acid residue by an amide bond.

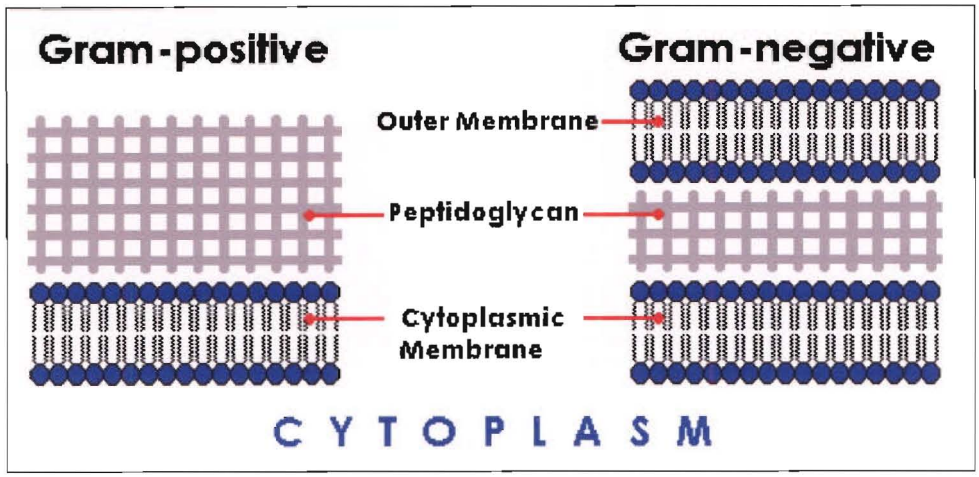

Figure 1.2 Diagramatic representation of Gram-positive and Gram-negative cell walls

The amino-acid composition of the peptide bridges, and degree of cross-linking, varies between bacterial species and consequently has been used for taxonomic purposes (Schleifer and Kandler, 1972). The typical composition of these peptide bridges is L-alanyl-γ-D-glutamyl-L-diaminoacyl-D-alanine. The third residue is diamino acid, however exact composition varies between bacterial species. In *Escherichia coli*, this residue is *meso*-diaminopimelic acid, in the pneumococcus (and *S. pyogenes*) it is L-lysine (Deibel and Seeley Jr, 1975, Severin and Tomasz, 2000). Adjacent glycan chains are cross-linked via these peptide bridges. Extracelluar peptide bonds are formed between the carboxyl group of the terminal D-alanine of one peptide, to an amino group of the diamino acid residue of an adjacent peptide. The energy required to catalyse this reaction comes from the cleavage of the terminal D-alanine residue. This is the penicillin-sensitive step in peptidoglycan biosynthesis (Wise Jr. and Park, 1965).

1.5.2.3 Peptidoglycan biosynthesis

Peptidoglycan synthesis can be divided into four stages (Goffin and Ghuysen, 1998), occurring in two locations. The first occurs in the cytoplasm. This is the formation of the peptidoglycan precursor UDP-N-acetylmuramyl-pentapeptide and UDP-N-acetylglucosamine. The second step involves the formation of the disaccharide-pentapeptide via a pyrophosphate bridge from the C_1 of N-acetylmuramic acid to a $C_{55}H_{89}$ undecaprenyl lipid II carrier. This intermediate facilitates the transfer of the precursor to the extracellular matrix (Koch, 2000a). The transport of the lipid-bound precursor occurs at a rate which matches nascent peptidoglycan synthesis, suggesting catalysis by as of yet unidentified proteins, suggested to be flippases (Holtje, 1998). Once external to the cytoplasmic membrane, the third stage of peptidoglycan biosynthesis takes place. This involves the transfer of the disaccharide pentapeptide from the lipid carrier to the growing glycan chain. This is the transglycosylation, which can occur by either of two mechanisms: (1)

displacement of pyrophosphate of the lipid II carrier by the 4-hydroxyl group of the terminal N-acetylglucosamine, or (2) displacement of the pyrophosphate of an existing glycan chain by the 4-hydroxyl group of the N-acetylglucosamine (Goffin and Ghuysen, 1998, Holtje, 1998). These reactions are catalysed by glycosyl transferases, and result in the linear growth of the glycan chain with repeated addition of disaccharide units (Goffin and Ghuysen, 1998).

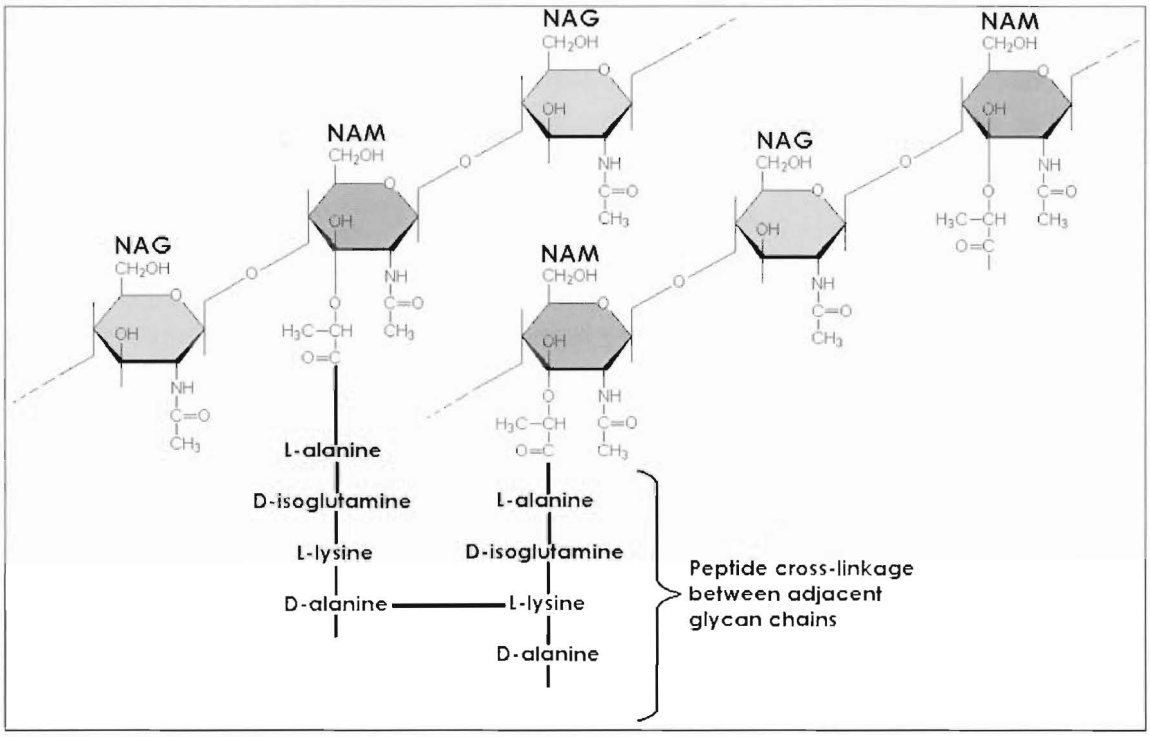

Figure 1.3 Molecular structure of pneumococcal peptidoglycan glycan chains of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), cross-linked by tetrapeptides of adjacent NAM resisdues.

The final stage of peptidoglycan biosynthesis involves the crosslinking of linear chains to form a strong, mesh like molecule. This reaction proceeds at the expense of the D-alanyl-D-alanine bond of the pentapeptide side chains. The group of enzymes which catalyse this reaction are D, D transpeptidases; a type of acyl serine transferase (Goffin and Ghuysen, 1998). Acyl serine transferases are so called due to their formation of an active site serine peptidyl enzyme complex. Assembly of this intermediate involves the liberation of the carboxy terminal D-alanine. The peptidyl moiety is next transferred to the diamino acid lysine residue of an adjacent peptide – and the cross-link is formed. The reaction catalysed by the D, D- transpeptidase is also the penicillin-sensitive stage of the process. Penicillin is structurally similar to the terminal D-alanyl-D-alanine of the pentapeptide and binds covalently to form a serine ester-linked penicilloyl enzyme. This intermediate is almost completely inert, the cross-linking capacity is therefore lost, and cell wall integrity compromised.

Because of the important role these enzymes play in the construction of the cell envelop, their inhibition is lethal to the cell. Penicillin is an analog of the natural substrate of these enzymes and consequently acts as an efficient inhibitor of their function (Wise Jr. and Park, 1965). Resistance to β-lactams in pneumococci has arisen due to alterations of the target PBPs that result in a reduction of their affinity toward the drugs, yet not impairing their biological function. Overall, pneumococcal resistance has been shown to be a function of the individual penicillin affinities of its PBPs (Zighelboim and Tomasz, 1980).

1.6 Epidemiology of Penicillin-Resistant *S. pneumoniae*

Strain identification is the process of analysing multiple isolates of a given species to identify whether they represent a single strain (clonal), or comprise of multiple strains (non-clonal). Identification systems are based on the premise that related isolates will share characteristics by which they can be differentiated from unrelated isolates. Such analyses are useful in several clinical settings. For example, a series of bacterial isolates from an individual patient may be examined to determine whether a relapsing condition is due to a recurring infection by a single isolate, or by sequential infections by different isolates. Bacterial strain identification also has utility in nosocomial infection control, when it is important to control outbreaks rapidly. In such a situation strain identification can help monitor the spread of the infection and identify the outbreak foci, allowing appropriate control measures to be taken. Outside the clinical milieu, strain identification is used to monitor the population dynamics of disease causing organisms in the community.

Strain identification systems can be conveniently grouped into phenotypic techniques, i.e., those that detect characteristics expressed by a microorganism, and genotypic techniques, i.e., those based on direct analysis of DNA.

1.6.1 Phenotypic identification techniques

Phenotypic methods vary in complexity and include techniques such as antimicrobial susceptibility, biotyping, phage typing, serotyping (Finland and Barnes, 1977), fatty acid profiling, SDS-PAGE (Pease et al., 1986), and multilocus enzyme electrophoresis (MLEE) (McDougal et al., 1992). Of these only serotyping has been vigorously utilized as an identification system for the pneumococcus. Although today serotyping has been largely superseded by molecular techniques, it still has a great deal of utility; especially when formulating vaccines which are currently based upon capsular polysaccharide types (Butler et

al., 1993). Furthermore, investigation of serotype prevalence has provided many important observations, for example: the prevalence of various serotypes shows temporal variation (Finland and Barnes, 1977); certain serotypes are responsible for the larger burden of invasive disease (Shapiro and Austrian, 1994); and that antibiotic resistance occurs most frequently in a narrow range of serotypes (Wust et al., 1995, Castaneda et al., 1997, Marchese et al., 2000, Soh et al., 2000, Maraki et al., 2001). For detailed epidemiological studies however, serotyping does not provide adequate discriminatory power. Isolates that share a common serotype may have quite different genetic backgrounds (Hall et al., 1996), consequently relatedness of isolates cannot necessarily be inferred from their serotype. Serotyping also has some short-fallings in that not all pneumococcal isolates have a distinguishable capsular type. Non-typable pneumococci are those that fail to produce a capsule. This is particularly common in pneumococci isolated from eye infections (Pease et al., 1986). Furthermore, on subculture in the laboratory, pneumococci have been shown to spontaneously stop capsule production, producing characteristic "rough" colonies.

1.6.2 Genotypic identification techniques

The worldwide emergence and dissemination of penicillin-resistant pneumococci during the 1980s and 1990s coincided with development of novel molecular biology techniques. The massive geographic spread of such resistance organisms was in fact a major motivation for the development of molecular surveillance techniques (Tomasz, 1999). Genotypic techniques of strain identification attempt to overcome some of the shortcomings of conventional phenotypic typing systems. Phenotypic techniques are based upon expressed cellular characteristics and traits; consequently they may yield variable or inconsistent results (e.g., an encapsulated pneumococcus that halts capsule production cannot be serotyped). Genotypic methods differ in that they utilise the bacterial genome itself as a molecular marker. Variations in the genomic DNA sequence of bacterial isolates are exploited to discriminate between individuals. Methods based on the DNA sequence of bacterial isolates have the advantage that all prokaryotic organisms have a DNA genome and are all therefore theoretically distinguishable.

The number of molecular typing methods that have been employed for the typing of prokaryotes is extensive. Some of the techniques used for typing of pneumococci include; amplified fragment length polymorphism (AFLP) (van Eldere et al., 1999), BOX PCR (van Belkum et al., 1996), enterobacterial repetitive intergeneric consensus (ERIC) based PCR (Hermans et al., 1995), multilocus restriction typing (MLRT) (Muller-Graf et al., 1999), multilocus sequence typing (MLST) (Enright and Spratt, 1998, Zhou et al., 2000) and chromosomal macro-

restriction profiling (MRP) using pulsed field gel electrophoresis (PFGE) (McEllistrem et al., 2000b) or field inversion gel electrophoresis (FIGE) (Lefevre et al., 1993). Each of the molecular typing methods described has it's own utility. Several reports have compared molecular methods based on typability, reproducibility, ease of use, cost and discriminatory power (Olive and Bean, 1999, van Belkum et al., 2001).

1.6.3 Molecular epidemiology of penicillin-resistant *S. pneumoniae*

Regardless of the choice of typing method, molecular typing can provide useful information, especially when considered in light of other phenotypic techniques. Most obviously, molecular typing of pneumococci has shown that within most serotypes there may be many genetically diverse organisms (Hall et al., 1996). These may be quite distinct bacterial lineages, although having in common the genes necessary for production of their given capsular type.

The application of molecular typing has done much to increase understanding of the evolution and dissemination of penicillin-resistant pneumococci. From such work it is evident that three distinct processes occur when resistance is developed within pneumococcal populations: (1) the acquisition of the resistance trait, and consequent "fine-tuning" of this trait; (2) the expansion of resistant clones; and (3) the transfer of the resistance trait between bacteria. Examples of each process are numerous in the literature. Analysis of resistant isolates of a discrete pneumococcal population from any given geographical area may aid in elucidating how such a resistant population developed (Hall, 1998).

In the first process, dubbed the "de novo" scenario (Tomasz, 1997), an organism sequentially acquires resistance determinants (altered *pbp* genes) resulting in a baseline increase in MIC. This eventually leads to the evolution of a small number of successful resistant clones, such as those documented by the pneumococcal molecular epidemiology network (PMEN) (McGee et al., 2001b). This is noted by the gradual increase of pneumococcal resistance in a given geographical area. The emergence of intermediate resistance strains will precede the highly resistant strains.

A sudden increase in the prevalence of resistant pneumococci in any geographically distinct region is characteristic of the second process. In contrast to the "de novo" scenario, the prevalence of resistance may jump from essentially zero to fairly high in a very short period of time. Such situations can usually be attributed to the importation and spread of a successful resistant clone. This is well demonstrated by the situation in Iceland documented in 1993

(Soares et al., 1993). In Iceland, the first penicillin-resistant pneumococcus was detected in December 1988. In the five years prior to this, no penicillin-resistant isolates had been detected. In the three years following the first isolation, the frequency of penicillin resistance isolates rose to 2.3%, 2.7% and 8.4% respectively. By the first quarter of 1992, 17% of isolates exhibited penicillin resistance (Kristinsson et al., 1992). Of these resistant-isolates, almost 70% belonged to serogroup 6 and had identical multi-resistant phenotypes. Fifty-seven such isolates were characterised by genomic MRP, MLEE, serotyping and PBP patterns. The Icelandic isolates were indistinguishable from a multi-resistant serotype 6B, which had been circulating in Spain for a decade previous.

The third process is the transfer of resistance between pneumococci and other streptococcal species. Oral streptococci have long been thought the most likely candidates for the transfer of DNA into the pneumococcus (Chalkley and Koornhof, 1990, Reichmann et al., 1997). Evidence suggests that resistant pneumococci may also donate DNA to viridans streptococci and impart resistance (Dowson et al., 1990). Analysis of the *pbp2b* gene nucleotide sequence of penicillin-resistant viridans streptococci identified a typical "mosaic" structure, of resistant blocks interspersed with sensitive blocks with high sequence identity to sensitive pneumococcal strain, R6 (Dowson et al., 1990). This supports the notion that a gene pool exists between streptococcal species, and that intra- and interspecies gene exchange may occur frequently *Streptococcus pneumoniae* (Reichmann et al., 1997, Hakenbeck et al., 2001).

1.6.4 Major penicillin-resistant pneumococcal clones

Perhaps the most successful resistant pneumococcal clones are those that originated in Spain, and subsequently began to disseminate worldwide. The intercontinental spread of a multi-resistant pneumococcal clone was first demonstrated by Munoz and colleagues (Munoz et al., 1991) in 1991. A serotype 23F clone resistant to penicillin recovered from children attending a day care centre in Cleveland, Ohio, was indistinguishable from a multi-resistant Spanish clone. Both strains were serotype 23F, had identical MLEE profiles, indistinguishable genomic MRPs and RFLP profiles of *pbp* genes. The Spanish clone was resistant to penicillin (MIC 1-2 μg/ml), chloramphenicol, co-trimoxazole and tetracycline. The Spanish 23F multi-resistant clone has subsequently been reported in many other parts of the world, including; France (Ferroni et al., 1996), Greece (Bogaert et al., 2000), Hong Kong (Ip et al., 1999), Korea (McGee et al., 1997, Tarasi et al., 1997), Taiwan (Shi et al., 1998), Columbia (Castaneda et al., 1998) and Mexico (Echaniz-Aviles et al., 1998).

Resistant pneumococcal clones have disseminated and spread throughout the world. PMEN is a group which was recently established to monitor the most globally widespread antibiotic-resistant pneumococcal clones (McGee et al., 2001b). To date 16 internationally spread resistant clones have been identified by PMEN, as listed in Table 1.4. These isolates have been defined by PFGE, BOX-PCR and MLST. Criteria for inclusion into the PMEN list includes; wide geographic distribution; strain well established over time; resistance to at least one clinically relevant antibiotic and description of the clone in international journals. Information regarding PMEN and the recognised clones can be acquired online (http://www.pneumo.com).

Clone designation	Year of Reporting	Serotype	Penicillin MIC (μg/ml)	Cefotaxime MIC (μg/ml)
Spain^{23F}-1	1991	23F/19F	2	0.5
Spain^{6B}-2	1992	6B	2	1
France^{9V}-3	1991	9V	2	1
Tennesse^{23F}-4	1995	23F	0.12	32
Spain^{14}-5	1996	14	2	1
Hungary^{19A}-6	1991	19A	2	0.5
South Africa^{19A}-7	1997	19A	0.5	0.5
South Africa^{6B}-8	1997	6B	0.5	0.25
England^{14}-9	1996	14	0.03	0.03
Slovakia^{14}-10	1995	14	8	1
Slovakia^{19A}-11	1995	19A	4	1
Finland^{6B}-12	1992	6B	1	-
South Africa^{19A}-13	1997	19A	8	2
Taiwan^{19F}-14	1998	19F	2	1
Taiwan^{23F}-15	1998	23F	1-2	1
Poland^{23F}-16	1999	23F	8	8

Table 1.4 Currently recognised pneumococcal clones as defined by PMEN.

The strain designations suggested by PMEN are not official strain designations, but rather the nomenclature of clonal groups as recommended by PMEN. However, as the PMEN group is comprised of many of the world's top pneumococcal researchers, it is likely these terms will receive widespread acceptance. Consequently these terms have been employed throughout this work to describe established international clonal groups.

1.7 Penicillin Resistance in *S. pneumoniae* in New Zealand

1.7.1 Penicillin resistance in invasive pneumococci

Pneumococci with reduced susceptibility to penicillin were first described in New Zealand in 1978 (Thornley et al., 1983). Co-ordinated nationwide surveillance of pneumococcal antibiotic resistance began at ESR in 1988 (Brett, 1996), although invasive isolates were screened for resistance prior to this (Heffernan, 1986). Among 233 invasive isolates submitted to ESR between 1981-1987, only intermediate resistance (MIC 0.1-1.0 μg/ml) was observed, with a prevalence of 1.3% during this period. Penicillin resistance (MIC ≥2.0 μg/ml) was first noted among invasive isolates in 1988. Between 1987 and 1995, penicillin resistance was noted in 0.4%, and intermediate penicillin resistance 1.1%, of submitted isolates.

1.7.2 Penicillin resistance in community acquired pneumococci

As with invasive isolates, penicillin-resistant pneumococci have remained uncommon in the community until relatively recently. A study performed at Green Lane hospital, Auckland, over eight months in 1974 yielded no penicillin-resistant pneumococci out of 130 isolates examined (Wood, 1976). However, low incidences of tetracycline and co-trimoxazole resistance were noted (6.9% and 3.9% respectively).

The proportion of penicillin-resistant pneumococci recovered in the community has been increasing yearly during the 1990s – with a particularly striking increase around 1993-94. This increase is best demonstrated by 1997 data from Riley et al (Riley et al., 1997), this group observed an increase in resistance among Auckland isolates from 0.8% in 1993 to 17% in 1994. This was accompanied by resistance increases in non-related antibiotics at the same time. From 1993-94, erythromycin, tetracycline and co-trimoxazole rose from 3 to 16%, 5 to 16% and 10 to 22% respectively. Other studies in 1997 suggest the overall rate of resistance (MIC ≥ 0.12 μg/ml) in New Zealand to be 17%, with Christchurch and Auckland having the highest prevalence (19%) (Brett et al., 1999).

1.8 Hypothesis and Aims

The incidence of pneumococci with reduced penicillin susceptibility has increased in New Zealand steadily since 1995. Based on this, the following hypotheses were considered;

(a) Observations and published reports from other parts of the world suggest that the rapid onset and spread of penicillin-resistant pneumococci are due to the introduction and

proliferation of a single successful clone. It is believed that this has occurred in New Zealand. Thus the first hypothesis tested was;

A single penicillin-resistant pneumococcal clone is responsible for the increased prevalence in resistance observed since 1995.

(b) The predominant clone is likely to have either developed *de novo* in New Zealand, or originated elsewhere in the world, and subsequently has been imported into New Zealand. The rapid emergence and increase in prevalence supports the latter scenario.

The prominent New Zealand resistant pneumococcal clone has been imported into the country from an exogenous source.

(c) A high level of resistance to cephalosporins is noted among New Zealand isolates. This may be associated with the major New Zealand clone, and if so, it may to have developed unique combinations of penicillin binding protein genes to impart this resistance.

The high-level cefotaxime-resistance is imparted by novel modifications within penicillin binding protein genes.

(d) Most resistant pneumococcal isolates recovered in New Zealand tend to be associated with very high-level resistance to macrolides, typically imparted by the *erm*(B) gene. The major New Zealand clone, therefore, is likely to harbour the *erm*(B) gene.

The high-level erythromycin resistance is imparted by the erm*(B) gene within New Zealand isolates.*

The aim of this thesis is to test each of these four hypotheses.

Chapter 2

Materials and Methods

2.1 Bacterial Strains and Growth Conditions

All clinical pneumococcal isolates used in this study were obtained from Medlab South, Christchurch, and the Institute of Environmental Science and Research Limited (ESR), Kenepuru Science Centre (KSC). A list of clinical isolates and their relevant phenotypes is given in appendix I. All reference strains are listed in Table 2.1. Descriptions of all media used are listed in appendix II.

Strain	Description	Source	Reference
ATCC 49619	NCCLS recommended antibiotic control strain	Medlab South Ltd, Christchurch.	(NCCLS, 1999)
ATCC 700669	Spain^{23F}-1	KSC, ESR.	(McGee et al., 2001b)
ATCC 700670	Spain^{6B}-2	KSC, ESR.	"
ATCC 700671	Spain^{9V}-3	KSC, ESR.	"
ATCC 51916	Tennessee^{23F}-4	KSC, ESR.	"
ATCC 700902	Spain^{14}-5	KSC, ESR.	"
ATCC 700673	Hungary^{19A}-6	KSC, ESR.	"
ATCC 700674	South Africa^{19A}-7	KSC, ESR.	"
ATCC 700675	South Africa^{6B}-8	KSC, ESR.	"
ATCC 700676	England^{14}-9	KSC, ESR.	"
ATCC 700677	Czech Republic^{14}-10	KSC, ESR.	"
RX1	R36A derivative	Dr Ann Holmes, Otago Dental School.	
R800	R36A derivative		

Table 2.1 Reference and control strains used in this study.

2.1.1 Cultivation and storage of bacterial strains

Pneumococcal strains were routinely cultivated on tryptic soya agar (TSA) supplemented with 5% sheep's blood. Samples were cultured for 18-24 h at 37°C in a 10% CO_2 incubator (primary isolation was performed under the same conditions in 5% CO_2). For long term storage cells

were removed after 18 h growth with a sterile cotton swab and suspended in brain-heart infusion (BHI) broth supplemented with 10% glycerol, and stored at -80°C.

2.2 Sample Isolation and Identification

Pneumococcal isolates were identified from clinical specimens using standard techniques. In all instances the following tests were performed for the identification of *S. pneumoniae*. As such, these tests were performed at either Medlab South (in the case of the Christchurch strains), or within the respective submitting laboratory (in the case of strains from KSC). Descriptions of these tests are included here for completion. Typically, colonies exhibiting characteristic pneumococcal morphology and α-haemolysis on TSA sheep's blood agar would be subcultured and tested to differentiate from other α-haemolytic streptococci. Differentiation consists of testing optochin sensitivity and bile solubility.

2.2.1 Optochin (Ethylhydrocupreine hydrochloride) sensitivity

A single pneumococcal colony was suspended in 1ml of Todd Hewitt broth. The cell suspension was spread three ways on to a pre-dried TSA plate containing 5% sheep's blood using a sterile cotton swab. The agar was allowed to dry for approximately 10 min before application of a 5 μg optochin disc (Oxoid, Hampshire, England). Plates were inverted and incubated for 18 h at 37°C in a 5% CO_2 incubator. After incubation the zones of inhibition were measured. A zone greater than 16mm indicated sensitivity and is a presumptive positive for *S. pneumoniae* (Ruoff, 1995).

2.2.2 Bile solubility

Results of the optochin sensitivity test were used in conjunction with the bile solubility test, performed using the plate method (Ruoff, 1995). A single, well-isolated colony was chosen from a 5% TSA plate containing a 18-24 h growth of the suspected *S. pneumoniae*. A loopful of 2% deoxycholate (pH 7.0) was placed directly on the colony, and the plate incubated aerobically for 30 min. A positive test was noted if the colony disintegrates under the drop.

2.3 Antimicrobial Susceptibility Testing

The majority of the antimicrobial susceptibility testing was performed at Medlab South, Christchurch, or the antimicrobial reference laboratory, KSC, Porirua. All pneumococcal isolates recovered at Medlab South from clinical specimens were routinely tested against a panel of three or four antibiotics depending on the site of infection. Typically isolates would be tested for sensitivity to oxacillin (1 μg), erythromycin (15 μg) and chloramphenicol (30 μg)

using antibiotic discs (Oxoid, Hampshire, England). Isolates recovered from sputa were tested with a different range of antibiotics including oxacillin (1 μg), erythromycin (15 μg), tetracycline (30 μg) and co-trimoxazole (25 μg). Any isolate that proved to have a decreased sensitivity to penicillin was tested further using Etest strips (AB-Biodisk, Solna, Sweden) to determine the isolate's MIC of penicillin and cefotaxime. At this stage disc sensitivities were also performed on any antibiotics not covered in the initial screen. Sensitivity to vancomycin (30 μg) was also tested by disc diffusion.

Isolates from Medlab South, and other NZ clinical labs, which were shown to have a decreased sensitivity to penicillin were submitted to the antimicrobial reference lab, KSC, for confirmation (during period 1997 - 2000, selection refined to cefotaxime-resistant isolates in 2001 due to high number of isolates being received). Confirmation included testing susceptibilities to cefotaxime, co-trimoxazole, erythromycin and penicillin by Etest, and tetracycline, chloramphenicol, and vancomycin by disc diffusion. Where available, sensitivities determined by the NZ antimicrobial reference laboratory were used preferentially over those of the submitting laboratory. For some isolates sensitivity data for specific antibiotics was not available, and other isolates with unusual phenotypes may have required independent verification. In such cases susceptibility testing was performed as stated in section 2.3.1 below. In each instance all tests were performed and interpreted according to the NCCLS guidelines (NCCLS, 1999).

2.3.1 Disc diffusion/Etest

Bacteria from an overnight culture were suspended in Mueller-Hinton broth to an optical density equivalent to a 0.5 McFarland standard. Mueller-Hinton agar plates supplemented with 5% sheep's blood, were dried for approximately 30 min before inoculation. The cell suspension was applied with a sterile cotton swab by streaking across the surface of the agar in three directions. The agar surface was allowed to dry for 10 min before application of discs or Etest strips. Typically no more than six discs, or two Etest strips, were applied to any plate. Disc zones were measured in millimetres and interpreted according to NCCLS standards (NCCLS, 1999). Etest results were read and rounded up to the next doubling dilution.

2.3.2 Agar dilution

Agar dilutions were performed when determining antibiotic MICs of a large number of strains, where testing by Etest would be impractical. The concentration range of each drug tested is shown in table 2.2 and followed a standard doubling dilution scheme (i.e. 0.06, 0.12, 0.25... 128, 256 μg/ml).

2.3.2.1 Preparation of antibiotic stocks

Antibiotics were weighed on an analytical balance, and a minimum of 20 mg of antibiotic powder transferred to a sterile universal tube. The powder was dissolved in an appropriate volume of a suitable diluent (Table 2.2) to give a final concentration of 2560 μg/ml (in the case of erythromycin a stock concentration of 5120 μg/ml was used), and adjusted to take into account the drug potency stated by the manufacturer. Antibiotics used were penicillin (Glaxo), piperacillin (Sigma), cephalothin (Lilly Research Laboratories), cefuroxime (Glaxo), cefaclor (Lilly Research Laboratories), cefotaxime (Roussel Ltd), meropenem (Astra Zeneca) and erythromycin (Sigma). Stocks of β-lactam antibiotics were not stored after use due to degradation. Erythromycin stocks were stored at -20°C

Antibiotic	Concentration range (μg/ml)	Diluent
Cefaclor	0.25 – 32.0	Phosphate buffer, pH 6.0
Cefotaxime	0.12 – 32.0	Water
Cefuroxime	0.25 – 32.0	Water
Cephalothin	0.25 – 32.0	Phosphate buffer, pH 6.0
Penicillin	0.12 – 8.0	Water
Piperacillin	0.25 – 32.0	Water
Meropenem	0.06 – 8.0	Water
Erythromycin	1.0 – 256.0	Ethanol/Water

Table 2.2 Antibiotics and dilutions used for susceptibility testing by agar dilution.

2.3.2.2 Preparation of test media

A suitable volume (17 ml per plate) of Mueller-Hinton medium was sterilised by autoclaving, and maintained at 50°C. The antibiotic stock solution was diluted to an intermediate concentration tenfold higher than the final concentration (volume of 2 ml per plate). After temperature equilibration of molten agar, the appropriate volume of antibiotic intermediate solution was added as well as 5% defibrinated sheep's blood (1 ml per plate). Container contents were then gently mixed and poured into petri dishes containing 20 ml of media per plate. Plates were stored at 4°C and were used within five days of preparation. Before inoculation of the plates, the plates were equilibrated at 37°C to allow the surface of the agar to dry.

2.3.2.3 Inoculation of test media

Colonies from an overnight growth (18 h) were suspended in sterile Todd Hewitt broth with a sterile cotton swab. The turbidity was adjusted to match that of a 0.5 McFarland standard (ca. 10^8 CFU/ml). Suspensions were further diluted 1:10 in sterile broth to give an adjusted concentration of 10^7 CFU/ml. An 100 µl aliquot of each suspension was transferred into an appropriate well of a 32 well seed plate. A replicating device was used to deliver inocula of all 32 isolates simultaneously. The replicator was calibrated to deliver 1 µl (ca. 10^4 CFU/ml) of suspension to the agar surface. Agar plates were inoculated beginning with the lowest drug concentration, and a control plate containing no antibiotic was included at the beginning and end of each series of antibiotic plates. Inoculated plates were allowed to stand until all inocula had been absorbed by the media, then were incubated overnight at 37°C in 5% CO_2.

2.3.2.4 Interpreting results of agar dilution plates

Before reading results, drug free control plates were checked to ensure the viability and purity of isolates. Quality control strain ATCC 49619 was also checked to ensure it yielded results within the acceptable accuracy ranges, as defined by the NCCLS standards (NCCLS, 1999). The MIC was defined as the lowest antibiotic concentration that inhibited visible growth.

2.4 Serotyping

Serotyping was performed by the Streptococcus Reference Laboratory, KSC, using the capsular reaction test (Neufeld test) and Danish system of nomenclature.

2.5 DNA Manipulations

Reagents and buffers used for various DNA manipulations are defined in appendix III.

2.5.1 DNA extraction

Unless stated otherwise, all DNA extractions used the rapid guanidine thiocyanate procedure described by Pitcher et al (1989). Growth from an 18 h TSA blood plate was collected using a sterile cotton swab and suspended in 1.0 ml of 0.9% saline. Cell suspensions were centrifuged for 1 min, 4°C, at 14800 x *g* and the supernatant discarded. The pellet was resuspended in 100 µl of fresh lysozyme (50 mg/ml) in TE buffer and incubated at 37°C for 30 min. Cells were lysed with the addition of 500 µl of GES reagent and incubated at ambient temperature for 5-10 min. Lysis was assessed by clearing of turbity, after which lysates were cooled on ice, and 250 µl of 7.5 M ammonium acetate was added. Samples were kept on ice a further 10 min, before

addition of 500 µl of 24:1 chloroform/IAA. The phases were mixed by inversion, and separated by centrifugation (10 min, 4°C, 14800 x *g*). The aqueous phase was transferred to a new tube, and DNA precipitated by addition of 0.5 volumes of ice-cold 2-propanol with gentle inversion. Tubes were incubated at ambient temperature for 10-15 min and DNA harvested by centrifugation (10 min, 4°C, 14800 x *g*). The DNA pellet was washed with 70% ethanol and air-dried. DNA was re-dissolved in 50 µl of sterile dH_2O supplemented with RNase A (20 µg/ml).

2.5.2 Agarose electrophoresis

DNA (including chromosomal DNA, PCR products and restriction digest products) was analysed by electrophoresis through 0.8-3.0% agarose gels. Gels were constructed by heating the appropriate amount of agarose (ultraPURE; Life Technologies) in 1x TAE buffer until the agarose melted. Gels were cast in perspex gel moulds using plastic combs to form wells. Typically, 8 µl of DNA sample was mixed with 1 µl of 6x bromophenol blue gel dye and loaded into each well. To verify fragment size, 3 µl of either 100 bp or 1 kb molecular weight marker (BioRad) was included in all gels. Electrophoresis was performed in a DNA sub cell apparatus (Jordan Scientific Co.) at approximately 6 V/cm.

Post electrophoresis, gels were stained in dH_2O containing 0.5 µg/ml ethidium bromide with gentle shaking for 15-20 min. Gels were visualised on a UV transilluminator (254 nm) and photographed with a Kodak DC120 electrophoresis documentation and analysis (EDAS) digital camera.

2.5.3 Polymerase chain reaction (PCR)

This section covers generic PCR, and applies to all the work presented in this thesis, with the exception of the PCR/DNA nucleotide sequencing performed for multilocus sequence typing, which required substantial "in house" sequencing. For consistency, the primers for the MLST scheme are listed below (table 2.3), but the method is outlined separately in section 2.6.

2.5.3.1 Preparation of PCR

PCR was performed in 0.5 ml thin-walled PCR tubes. Each reaction consisted of the appropriate primers at a final concentration of 2 pmol per reaction; each dNTP (dATP, dCTP, dGTP, dTTP) at a final concentration of 200 µM; 10% of the total reaction was 10x PCR buffer, 2.5 units of *Taq* DNA polymerase and sterile dH_2O to bring the reaction volume to 50 µl. 10x PCR buffer, dNTPs, and *Taq* polymerase were all obtained from Boehringer Mannheim/Roche. Primers (tables 2.3-2.6) were purchased from Gibco/Life Technologies. Reaction mixtures were overlaid

with 2 drops of mineral oil (Sigma). Primers for MLST (Table 2.3) were as described by Enright and colleagues (1998).

Amplification was carried out in a Corbett Research FTS 320 thermal cycler. Amplification consisted of an initial denaturation of 1 min at 94°C, then 35 cycles for the following three step cycle; template denaturation for 15 sec at 94°C, primer annealing for 45 sec at 55°C and template elongation for 1 min at 72°C. A final extension step of 5 min at 72°C was included, followed by an ongoing soak step at 4°C to refrigerate reactions until processing. The presence of PCR amplification products (amplicons) was verified by electrophoresis through 1% agarose gels.

Primer name	Nucleotide sequence	Target gene
aroE-up	5'-gcc ttt gag gcg aca gc-3'	shikimate dehydrogenase
aroE-dn	5'-tgc agt tca (g/a)aa aca t(a/t)t tct aa-3'	
gdh-up	5'-atg gac aaa cca gc(g/a/t/c) ag(c/t) tt-3'	glucose-6-phosphate dehydrogenase
gdh-dn	5'-gct tga ggt ccc at(g/a) ct(g/a/t/c) cc-3'	
gki-up	5'-ggc att gga atg gga tca cc-3'	glucose kinase
gki-dn	5'-tct ccc gca gct gac ac-3'	
recP-up	5'-gcc aac tca ggt cat cca gg-3'	transketolase
recP-dn	5'- tgc aac cgt agc att gta ac-3'	
spi-up	5'-tta ttc ctc ctg att ctg tc-3'	signal peptidase I
spi-dn	5'-gtg att ggc cag aag cgg aa-3'	
xpt-up	5'-tta tta gaa gag cgc atc ct-3'	xanthine phosphoribosyl transferase
xpt-dn	5'-aga tct gcc tcc tta aat ac-3'	
ddl-up	5'-tgc (c/t)ca agt tcc tta tgt gg-3'	D-alanine-D-alanine ligase
ddl-dn	5'-cac tgg gt(g/a) aaa cc(a/t) ggc at-3'	

Table 2.3 PCR primers used for the amplification and sequencing of housekeeping genes for multilocus sequence typing.

Primer name	Nucleotide sequence	Target gene	Reference
*pbp1a*1827	5'-tgg gat gga tgt tta cac aaa tg-3'	*pbp1a* gene	(Asahi and Ubukata, 1998)
*pbp1a*3098	5'-tgt gct ggt tga tga gga ttc tg-3'		
pbp2b-seq	5'-gag a(g/c)t tga cgc ctg att cct t-3'	*pbp2b* gene	This study
*pbp2x*958	5'-tat gaa aag gat cgt ctg gg-3'	*pbp2x* gene	(Asahi et al., 1999)
*pbp2x*2105	5'-aga gag tct ttc ata gct gaa gc-3'		

Table 2.4 PCR primers used for amplification and sequencing of penicillin binding protein genes.

Primer name	Nucleotide sequence	Target gene	Reference
Pn1Aup	5'-cgg cat tcg att tga ttc gct tct-3'	*pbp1a* gene	(Coffey et al., 1991)
Pn1Adown	5'-ctg aga aga tgt ctt ctc agg-3'		
pbp1b-up	5'-gtg gta taa tag ata aag tga gg-3'	*pbp1b* gene	(Hakenbeck et al., 1998)
pbp1b-dn1[a]	5'-ccc ttg aag aag aag gtc g-3'		
pbp1b-dn2[b]	5'-ccc ttg aca tca aca ccc-3'		
pbp2a-up	5'-gtg aac tag agg act ctg-3'	*pbp2a* gene	(Hakenbeck et al., 1998)
pbp2a-dn	5'-gaa ata gat tga cta tcg-3'		
Pn2Bup	5'-gat cct cta aat gat tct cag gtg g-3'	*pbp2b* gene	(Dowson et al., 1989b)
Pn2Bdown	5'-caa tta gct tag caa tag gtg ttg g-3'		
pbp2x-up	5'-cgt ggg act att tat gac cga aat gg-3'	*pbp2x* gene	(Munoz et al., 1991)
pbp2x-dn	5'-aat tcc agc act gat gga aat aaa cat att a-3'		

Table 2.5 PCR primers for the amplification of *pbp* genes.
Amplified fragments were subsequently used for RFLP typing (primers Pn2Bup and Pn2Bdown were also used for determination of DNA sequence), *(a)* designed based on *S. pneumoniae* R6 DNA sequence, *(b)* designed based on cefotaxime-resistant R6 transformant $R6_{T5}$-CCC (Hakenbeck et al., 1998).

Primer name	Nucleotide sequence	Target gene	Reference
ermAM-1	5'-tca acc aaa taa taa aac aa-3'	*ermB* gene	(Shortridge et al., 1996)
ermAM-2	5'-aat cct tct tca aca atc ag-3'		
mefE-1	5'-agt atc att aat cac tag tgc-3'	*mefE* gene	(Sutcliffe et al., 1996a)
mefE-2	5'-ttc ttc tgg tac taa agt gg-3'		

Table 2.6 PCR primers for amplification and sequencing of macrolide resistance genes.

2.5.3.2 Restriction digestion of PCR products

PCR amplicons of the *pbp* genes (Table 2.5) were typically analysed by restriction endonuclease digestion. Restriction endonucleases used in this work were purchased from Roche, and used as per manufacturer's instructions. Typically restriction digests were performed in 15 µl and incubated at the appropriate temperature for 3 h. The enzymes used for this work were *Dde*I (C'TNAG), *Hin*fI (G'ANTG) and *Rsa*I (CT'AG), (N represents any one of the four nucleotides; A,T,G or C).

2.5.3.3 DNA sequencing of PCR products

With the exception of the sequencing performed for MLST (section 2.6), DNA sequencing was sent to the University of Waikato DNA sequencing facility. DNA fragments for sequencing consisted solely of PCR products and were sequenced using the identical primers for the initial PCR amplification. Unincorporated primers and nucleotides were removed from DNA templates using the QIAquick spin column PCR purification kit (Qiagen).

DNA sequencing was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems) using BigDye terminators (Applied Biosystems) at the the University of Waikato, Hamilton, New Zealand. The nucleotide sequence of both DNA strands was determined and sequence alignments were performed using Clustal X (Thompson et al., 1997).

2.5.4 Pulsed field gel electrophoresis (PFGE)

2.5.4.1 Preparation of agarose embedded chromosomal DNA

Bacterial growth from an 18 h TSA blood plate was collected using a sterile cotton swab and suspended in 3 ml of PETT IV buffer to give a turbidity equivalent to a 3.0 McFarland standard. The cells were collected by centrifugation for 3 min, 4°C at 6600 x *g*. The supernatant was discarded and pellet resuspended in 125 µl of PETT IV buffer. A 1.6% solution of pulsed field certified agarose (Bio-Rad) in dH_2O was heated until dissolved and held at 50°C in a water bath. Plugs were formed by rapidly mixing 240 µl of molten agarose with the cell suspensions and injecting into plug moulds (Bio-Rad). Plugs were allowed to solidify (10-15 min), then placed into sterile universal bottles containing 2 ml EC lysis buffer with lysozyme (final concentration 1 mg/ml) and heat-treated RNase (final concentration 20 µg/ml) and incubated overnight at 37°C in a shaking water bath. The lysis buffer was subsequently removed after 24 h and replaced with 2 ml ESP buffer containing proteinase K (final concentration 0.5 mg/ml) and incubated overnight at 50°C. Following incubation plugs were rinsed by soaking in 20 ml dH_2O at ambient temperature for 30 min. Plugs were transferred to sterile plastic 50 ml tubes and were washed twice by incubation in 1x TE buffer overnight at 4°C. Plugs were subsequently cut into 6 even pieces and stored at 4°C in 1 ml of 1x TE until required.

2.5.4.2 Restriction endonuclease digestion of agarose embedded DNA

Plug slices were digested using either of the enzymes *Sma*I or *Apa*I (Roche). Digestions were performed in sterile 1.5 ml Eppendorf tubes and consisted of 20 units of respective enzyme with 15 µl of appropriate digestion buffer in 135 µl of sterile dH_2O per reaction. Reactions were incubated overnight at 25°C (*Sma*I) or 30°C (*Apa*I).

2.5.4.3 Electrophoresis of agarose embedded DNA

An 1% agarose gel was prepared by heating the appropriate amount of pulsed field certified agarose (either 1.0 or 1.5 grams) in the corresponding volume of 0.5x TBE buffer (either 100 or 150 ml respectively). The gel was cast and DNA embedded agarose plugs were loaded into the wells. Once loaded the wells were sealed with 1% agarose to prevent further movement of the

plugs. Concatamers of lambda DNA (PFG marker, New England Biolabs) was used as a size standard. Electrophoresis was performed on both CHEF mapper and CHEF DRIII (both Bio-Rad) systems. Both systems required 2 L of 0.5x TBE running buffer. Electrophoretic conditions were as follows; running temperature 14°C, gradient 6.0 V/cm, run time 22 hours, initial switch time 5 sec, final switch time 35 sec and a linear ramping factor. Following electrophoresis, gels were stained in ethidium bromide (05 μg/ml) for 30 minutes, and destained in dH_2O for up to an hour. Gels were visualised on a UV transilluminator (254 nm) and image obtained using a Kodak DC120 digital camera.

2.5.4.4 Interpretation of banding patterns

*Sma*I digests of pneumococcal chromosomal DNA produces approximately 15 fragments, ranging in size from 25–500 kbp. Banding patterns were compared visually and were interpreted essentially as described by Tenover *et al* (1995), with some modifications. Tenover *et al* suggest that patterns differing in up to three bands from an index pattern can be considered highly related, and those differing in up to six bands considered possibly related. As the current investigation was spread temporally over five years, it was decided that all "possibly related" profiles were to be considered as belonging to the same macro-restriction group. Each group was numbered arbitrarily as new profiles were observed, e.g., 1, 2, 3 etc. Within each group, identical profiles were considered distinct restriction types, and distinguished with a uppercase letter, e.g. 1A, 1B, 1C etc. Therefore, by definition; all isolates with MRP 1A would be genetically indistinguishable and considered to belong to the same clonal group; two isolates with MRPs 1A and 1B respectively, would be designated to the same group and considered related; and two isolates with MRPs 1A and 2A would be designated to different groups and considered unrelated. Without the ability (in most instances) to discern an index case, types are considered to belong to a given group if they differ by no more than six bands from the most similar member of the cluster, as suggested by Hall (Hall, 1998).

2.5.5 Southern hybridisation

All Southern hybridisations were performed using the non-radioactive digoxygenin-dUTP (DIG) DNA labelling and detection kit (Roche). All buffers and solutions used for Southern hybridisations are described in Appendix III.v.

2.5.5.1 Probe labelling

Probes were created using the PCR DIG probe synthesis kit (Roche) as per manufacturer's instructions. PCR labelling reactions were performed in 50 μl volumes. The resultant PCR

product was purified using the QIAquick spin column PCR purification kit (Qiagen). Prior to use, the DIG-labelled DNA probe was denatured by boiling for 5 min, and rapidly cooled on ice.

2.5.5.2 Southern transfer

DNA separated by PFGE was transferred to a positively charged nylon membrane (Roche) using a vacuum transfer unit (Hoefer TransVac TE80). A constant suction of 70 mm Hg was obtained with an Air Cadet vacuum/pressure station (Cole-Palmer). Transfer was conducted in accordance with manufacturer's (Hoefer) protocol.

Briefly, after assembling the transfer unit and placement of both gel and membrane, the gel was treated sequentially with approximately 30 ml of depurination, denaturation and neutralisation solutions. Solutions were pipetted onto the gel in sufficient volume to cover the surface and left for 20 min under constant vacuum. Liquid was removed by pipette before treatment with subsequent solution. Following the neutralisation solution, 20x SSC buffer was added in sufficient volume to cover the entire gel, and transfer was performed for 120 min. Following transfer, the agarose gel was re-stained and examined on an UV transilluminator to estimate the efficiency of transfer. The membrane was removed from the TransVac and the transferred single strand DNA was fixed to the membrane by exposure to a UV light source for 30 sec, at 1200 kJ in an UVC-515 Ultraviolet multilinker (Ultra-lum).

2.5.5.3 Probe hybridisation

Hybridisation was conducted as per the manufacturer's instructions (Roche). Briefly, the membrane was placed in a Hybaid glass tube, and prewarmed at 42°C with 25 ml (for PFGE gel 14x15 cm membrane) of hybridisation buffer (DIG Easyhyb, Roche). Prehybridisation was performed for 30 min in a Micro-4 HB-MCR4 hybridisation oven (Hybaid). Following prehybridisation, the buffer was discarded and replaced with 7.3 ml of hybridisation solution containing the denatured DIG-labelled probe. Hybridisation was performed at 42°C overnight (16 h) with constant rotation. Post-hybridisation, the solution containing the DIG labelled probe was decanted and stored at -20°C for future use. The membrane was subject to two 5 min low stringency washes (2x SSC, 0.1% SDS at ambient temperature) and two 15 min high stringency washes (0.5% SSC, 0.1%SDS at 68°C).

2.5.5.4 Signal detection

Hybridised DIG labelled DNA probes were detected as per manufacturer's instructions (Roche). The membrane was rinsed in approximately 100 ml of washing buffer for 5 min at ambient temperature. The membrane was next incubated in blocking solution (30 min), antibody (anti-digoxigenin-alkaline phosphatase conjugate) solution (30 min), washing buffer (2x 15 min) and detection buffer (5 min). The membrane was sealed in plastic with approximately 1 ml of CSPD (chemiluminescent substrate for alkaline phosphatase) and incubated at 37°C for 15 min. Chemiluminescence from the membrane was detected on hyperfilm (Amersham Pharmacia Biotech) after a 5 min exposure.

2.5.5.5 Stripping/Re-probing membranes

The DIG-11-dUTP is alkaline labile, therefore efficient stripping of the blots was easily achieved. This was done by first washing the membrane with dH_2O, before subjecting it to two 15 min washes at 37°C in 0.2 M NaOH containing 0.1% SDS. This is followed by a 5 min rinse in 2x SSC at which stage the membrane is ready to be re-probed.

2.6 DNA Sequencing

Sections 2.6.1 through to 2.6.3 refer to "in house" sequencing performed at the Kenepuru Science Centre. Preparation of PCR products for sequencing at the University of Waikato DNA sequencing facility was covered in section 2.5.3.3.

2.6.1 PCR amplification of internal gene fragments

Template DNA for the initial PCR was extracted using the InstaGene Matrix kit (BioRad) according to manufacturer's directions. DNA preps were stored -20°C. PCR was performed in 0.2 ml thin-walled PCR tubes in a total reaction volume of 50 ml. The PCR reaction mixture contained; 6 µl template DNA, 1 µl of each of the appropriate primers (Table 2.3) at a final concentration of 0.8 pmol and 42 µl Platinum PCR supermix (Gibco).

PCR amplification was performed in a Perkin-Elmer Cetus thermocycler. After an initial denaturation step of 5 min at 94°C, amplification consisted of 35 cycles, three steps per cycle: template denaturation for 30 sec at 94°C, primer annealing for 30 sec at 50°C and elongation for 30 sec at 70°C. A final extension step of 5 min at 70°C was also included followed by a final soak at 4°C. The presence of a reaction product was verified by subjecting 5 µl of the PCR

reaction mixture to electrophoresis through a 2% agarose gel at 100 V for 1 h. Gels were stained with ethidium bromide and visualised on a UV transilluminator.

PCR products were cleaned using the QIAquick 96-well plate PCR purification kit (Qiagen) as per manufacturer's instructions. This procedure also required the QIAvac 96 (Qiagen) and vacuum source. Purified DNA was stored at -20°C.

2.6.2 Sequencing reaction

To ensure sequence data was as accurate as possible, the nucleotide sequence of both DNA strands was determined. This is accomplished by setting up two reactions for each gene, each using only one of the two primers. As such, each strain required 14 sequencing reactions. With each batch of sequencing reactions performed a control was included to ensure the integrity of the sequencing reaction. The control recommended by Perkin Elmer, the plasmid pGEM with primer M13, was used. Template DNA concentration was determined by comparison with a low molecular mass DNA ladder (LifeTechnologies) on a 2% agarose gel as per manufacturer's instructions.

Sequencing reactions were performed in 20 µl volumes and contained; 0.5-2.0 µl of purified template DNA to ensure a final concentration of 5 ng/µl, 1 µl of each single primer (3.2 pmol), 4 µl of BigDye terminator mix (Applied Biosystems) and sufficient dH_2O to bring the total reaction volume up to 20 µl. The sequencing reactions were performed in a Perkin-Elmer Cetus thermocycler. Dye incorporation consisted of 25 cycle reaction, three steps per cycle: template denaturation for 10 sec at 94°C, primer annealing for 5 sec at 50°C and elongation for 2.5 min at 60°C. A final soak at 4°C was also included. At the completion of the reaction, products were stored at -20°C until ready to purify for sequencing.

2.6.3 Preparation of product for sequencing

Prior to sequencing, un-incorporated dye-terminators were removed by ethanol precipitation. This was performed by firstly adding 80 µl of dH_2O to the 20 µl sequencing reaction product. In a new 0.5 ml tube, 10 µl of 3 M sodium acetate (pH 4.6) and 250 µl of 100% ethanol (EtOH) was added. The diluted sequencing reaction mixture was pipetted into the EtOH and mixed thoroughly. Tubes were incubated at ambient temperature for 15 min to precipitate the extension products, before collecting DNA by centrifugation (25 min, 14800 x *g*). The supernatant was carefully discarded and pellet washed with 250 µl of 70% EtOH. Again the DNA was collected by centrifugation (25 min, 14800 x *g*) and the supernatant carefully removed. The resulting

pellet was incubated at 37°C and allowed to air dry. Pellets could be stored at -20°C at this stage.

To complete processing, pellets were resuspended in 15 µl of template suppression reagent (TSR, Applied Biosystems) and mixed. The samples were heated in a hot block at 95°C for 2 min to denature the DNA and immediately placed on ice. At this stage the samples were loaded into the ABI Prism 310 automated sequencer (Applied Biosystems) and sequenced using the long capillary and a gel temperature of 50°C, in accordance with manufacturer's instructions.

2.7 Protein Manipulations

Sections 2.7.1 – 2.7.3 based on protocol of Zhao, with minor modifications (Zhao et al., 1999).

2.7.1 Bacterial membrane preparations

Pneumococcal cells were grown in 500 ml of brain heart infusion medium to an OD_{600} of 0.5 - 0.8. Cells were harvested by centrifugation (4400 x *g* for 8 min at 4°C) and the pellet washed with phosphate extraction buffer. Cells were resuspended in 5 ml extraction buffer and subject to mechanical lysis by sonication (Vibra-cell, Sonics). Cell suspensions were pulsed twice with a sonicator (each pulse 75% amplitude, one min bursts), with ice bath cooling.

Crude cell debris was removed by centrifugation at 12000 x *g* for 10 min at 4°C. Supernatant fractions were collected and membrane proteins collected by centrifugation at 150 000 x *g* for 35 min (RC-M150GX ultracentrifuge, Sorvall). Pellets were collected, washed once, and resuspended in 500 µl of extraction buffer. Protein concentrations were estimated using the BioRad protein assay kit as per manufacturer's instructions. Protein extracts were stored at -80°C until further use.

2.7.2 Labelling of penicillin binding proteins with Bocillin FL

Penicillin binding proteins were detected using Bocillin FL, a commercially prepared fluorescent penicillin analogue (Molecular Probes, Inc.). Labelling was performed in 50 µl volumes containing; 37.5 µl membrane preparation (~150 µg of protein) and 25 µl of Bocillin FL solution (final concentration range 0.4-50 µM). Reaction mixtures were incubated at 35°C for 30 min. After labelling, 50 µl of sample buffer was added, and the reaction mixture incubated at 100°C for 3 minutes. Sample volumes of 5-10 µl (7.5-15 µg protein) were subjected to SDS-PAGE analysis.

2.7.3 SDS-PAGE separation of labeled PBPs

Separation of protein bands was achieved by electrophoresis through 7.5% polyacrylamide gels using a BioRad Mini PROTEAN II electrophoretic unit under a constant current of 40 mA. Polyacrylamide gels prepared as stated in appendix III.iv.ii. Labeled protein bands were visualized with the BioRad FluorS Multimager.

Chapter 3

Population Dynamics of Antibiotic-Resistant Pneumococci in Christchurch

3.1 Introduction

Christchurch is the largest centre in the South Island of New Zealand, with a population of around 340 000. It is situated on the east coast (Figure 4.1), and provides an international entry point for the South Island. During the 1990s New Zealand observed an increase in antibiotic resistance among *S. pneumoniae*. Resistance to penicillin increased from 1.8% in 1988-90 to 19% in 1997-99 among non-invasive pneumococci, and from 1.0% in 1988-90 to 15% in 1997-99 among invasive isolates (Brett and Ellis-Pegler, 2001). The prevalence of antibiotic resistance in Christchurch is among the highest in the country. A study of pneumococci isolated from four New Zealand cities during 1997 found the prevalence of high-level penicillin resistance (MIC $\geq$ 2 μg/ml) to be 10% in Christchurch. By comparison, the incidence in the other centres was 6%, 5% and 0% in Auckland, Hamilton and Wellington respectively (Brett et al., 1999).

Medlab South is a Christchurch community medical laboratory, providing routine diagnostic testing for medical practitioners in Christchurch, and the greater South Island area. As part of their routine susceptibility testing, increased antibiotic resistance in *S. pneumoniae* has been noted over the past decade (Figure 3.1). In particular, pneumococci with reduced susceptibility to penicillin have been recovered with steadily increasing frequency since 1993. In little over four years, from late 1994 to early 1999, the incidence of non-susceptible pneumococci rose from 3% to 30% of those recovered. This rapid increase in prevalence of resistant pneumococci is reminiscent of overseas reports of rapid increases in resistance. The often-cited Icelandic experience is perhaps one of the most convincing examples (Soares et al., 1993). Resistant pneumococci had not been observed in Iceland prior to 1988, yet by 1992 prevalence was at 17% (Kristinsson et al., 1992). France is another example; resistant pneumococci were rare ($\leq$ 1.1%) until 1987 (Geslin et al., 1992), but increased steadily thereafter to reach 25% prevalence in 1993 (Ferroni et al., 1996). Such observations are not limited to Europe. In Hong Kong,

pneumococci with reduced penicillin susceptibility were first reported in 1993; by 1995 their prevalence was observed to be 28.9% (Kam et al., 1995).

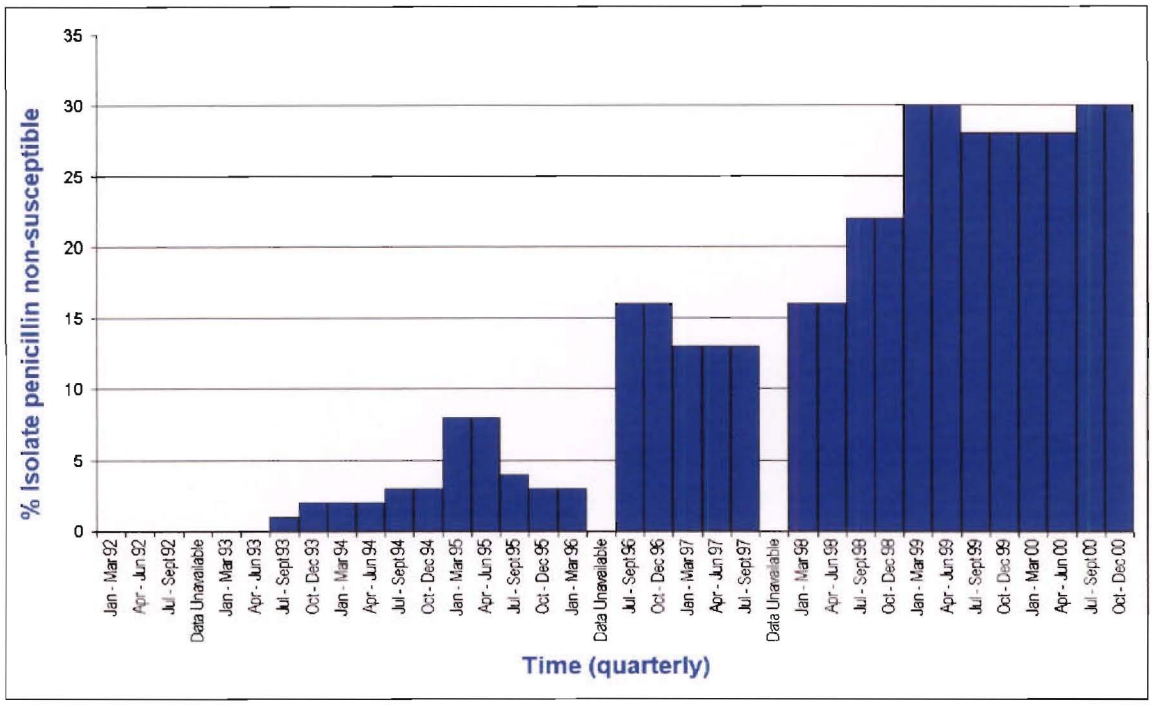

Figure 3.1. Proportion (%) of all pneumococcal isolates recovered at Medlab South, Christchurch, between January 1992 – December 2000, with a reduced susceptibility to penicillin (MIC $\geq$ 0.12 μg/ml)

In cases of dramatic increases in resistance rates during a short period of time, detailed molecular analysis of the pneumococcal isolates often uncovers that a significant proportion of the resistant population are clonally related. In each of the cases previously described (Iceland, France and Hong Kong) resistant pneumococcal clonal lineages were found to be largely responsible for the increase in prevalence. In France, 65% of resistant isolates were found to belong to serotype 23F, and the 23F isolates were genetically related as assessed by macro-restriction profile (MRP) analysis (Ferroni et al., 1996). The predominant resistant serotypes in Hong Kong were 23F and 19F. Molecular analysis by MRP of 105 resistant isolates detected a major clonal group which comprised 74% of the isolates, and which were indistinguishable from the globally widespread Spanish^{23F}-1 clone. However, in Hong Kong this lineage was found to express capsular types 23F, 19F and 14 (Ip et al., 1999).

Based on overseas observations such as those described, it was likely that the recent rapid increase of pneumococcal resistance in Christchurch is due to the dissemination of a resistant clone. To test this hypothesis, resistant pneumococci were collected over a five-year period and

MRPs of genomic DNA were determined. Profiles were compared visually to cluster closely related isolates.

3.2 Results

Between 1997 and 2001, 200 pneumococcal isolates with reduced susceptibility to penicillin were isolated and included in this study. The majority of these isolates were obtained during two sampling periods; August 1997 through to May 1999 (163 isolates), and March 2001 through to July 2001 (34 isolates). Three random isolates from October 2000 were also included. The patients from whom the isolates were recovered ranged in age from one week to 91 years. Ninety-one isolates (45.5%) were recovered from patients two years or younger, and 34 (17.0%) recovered from patients 60 years or greater. Isolates were recovered from 104 (52.5%) females, 94 (47.5%) males, with the sex of two patients unknown. The main body sites of isolation are shown in table 3.1. The most prominent sites of isolation were ears, eyes and sputa – combined accounting for 78.6% of the isolates. As the pneumococcus is commonly associated with infections at such sites, it is not unreasonable to assume that the recovered isolates were significant, and the agent responsible for the consultation. The same cannot necessarily be said for the other sites of isolation (e.g., nasal/throat), which may correspond to asymptomatic carriage of the organism. However, such isolates were included in this study and may consequently approximate the level of carriage of resistant pneumococci in the community.

Isolation site	Number of isolates	%
Ear	59	29.5
Eye	56	28.0
Sputum	42	21.0
Nasal	19	9.5
Adenoid tissue	10	5.0
Throat	4	2.0
Other	10	5.0

Table 3.1 Body sites from which penicillin non-susceptible pneumococci were recovered.

3.2.1 Antimicrobial susceptibilities

The criteria for inclusion in this study was a penicillin MIC of 0.12 μg/ml or greater. The highest penicillin MIC recovered was 8.0 μg/ml, and the MIC_{50} and MIC_{90} were 2 and 4 μg/ml, respectively. Including resistance to penicillin, a further 91.5% (183) were also resistant to co-trimoxazole, 79.5% (159) resistant to erythromycin, 75.5% (151) resistant to tetracycline and

16.5% (33) resistant to chloramphenicol. No resistance to vancomycin was observed. Multi-drug resistance, as defined as being resistant to three non-related antimicrobial agents (including β-lactams), was observed in 80.5% (161) of the isolates. The most frequently observed multi-resistant phenotype imparted resistance to co-trimoxazole, erythromycin, tetracycline, as well as reduced penicillin susceptibility. This phenotype was noted in 121 (60.5%) of the isolates.

3.2.2 Typing by DNA macro-restriction analysis

The 200 pneumococcal isolates could be divided into 26 groups by DNA MRP analysis using the enzyme *SmaI* and pulsed field gel electrophoresis (PFGE). Each of these groups could be sub-divided into a total of 59 subgroups. The four largest groups (Groups 1, 2, 3 and 11) accounted for 171 (85.5%) of the isolates (48.5%, 12.5%, 12% and 12.5% for each group respectively). Groups 1, 2 and 3 (Figure 3.2 and Table 3.2) are comprised of pneumococcal isolates with intermediate to high penicillin resistance (MIC 0.5-8.0 μg/ml), whereas members of group 11 are strictly of intermediate penicillin resistance (MIC 0.12 μg/ml) although multiply resistant to other classes of antibiotics (Table 3.4).

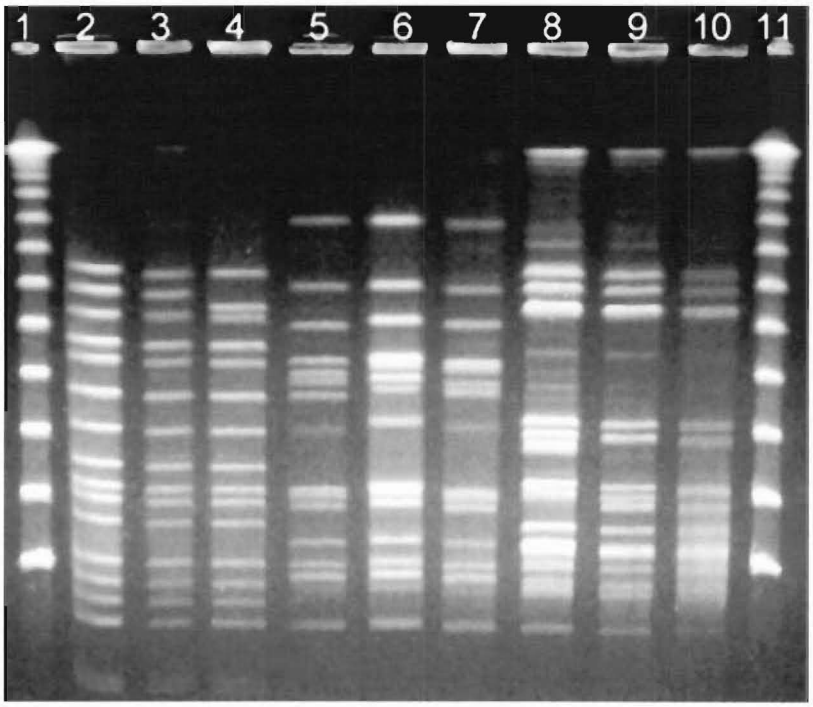

Figure 3.2 Comparison of the three most predominant DNA MRPs (groups 1, 2 and 3) belonging to Christchurch isolates of *S. pneumoniae* with reduced susceptibility to penicillin.
Lanes 1) Lambda ladder, 2) isolate Sp013 (type 1A), 3) isolate Sp019 (type 1A), 4) isolate Sp028 (type 1C), 5) isolate Sp007 (type 2P), 6) isolate Sp020 (type 2A), 7) isolate Sp035 (type 2A), 8) isolate Sp041 (type 3A), 9) isolate Sp009 (type 3E), 10) isolate Sp211 (type 3C), 11) Lambda ladder.

The most prominent group contained 97 isolates (48.5%), and five different MRPs (Figure 3.3). Of these, 91 of the *Sma*I profiles were indistinguishable (type 1A) and were thus considered clonal. To further assess the relatedness of this group, a subset of 42 isolates were digested with the restriction endonuclease *Apa*I and the resulting DNA fragments separated by PFGE. Each of the isolates yielded indistinguishable MRPs (Figure 3.4). Each of the group 1 isolates analysed had the same antimicrobial resistance pattern – all were resistant to co-trimoxazole, erythromycin and tetracycline as well as having reduced susceptibility to β-lactams. MIC's to penicillin and cefotaxime ranged from 1-8 μg/ml and 0.5-16 μg/ml, respectively. All isolates of this group were susceptible to chloramphenicol and vancomycin. Fourteen of the isolates belonging to this group were serogrouped and determined to belong to serogroup 19, four of the 14 were typed further as 19F. Unfortunately, serotype data for all isolates was unavailable. This is because only one laboratory in New Zealand (the Streptococcus reference laboratory, KSC, ESR) has the capacity to serotype pneumococci. Serotyping is restricted to all invasive isolates and a subset of penicillin-resistant isolates. Nevertheless, using the limited serotyping information available in conjunction with the molecular typing data, it is possible to tentatively assign serotypes to major clonal groups.

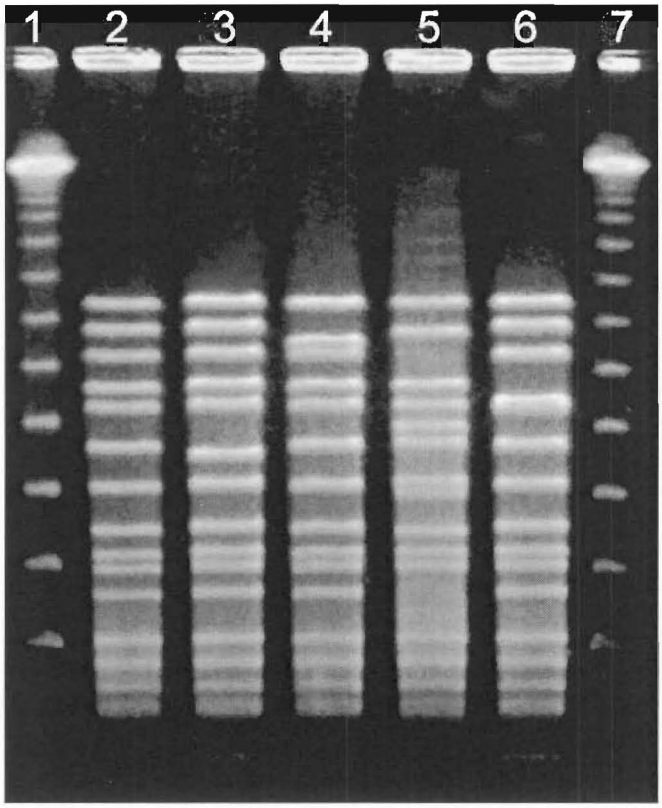

Figure 3.3 Comparison of the *Sma*I DNA macro-restriction subtypes of the major group 1 profile predominant among Christchurch penicillin non-susceptible isolates of *S. pneumoniae*.
Lanes 1) Lambda ladder, 2) isolate Sp003 (Type 1A), 3) isolate Sp269 (Type 1B), 4) isolate Sp028 (Type 1C), 5) isolate Sp221 (Type 1D), 6) isolate Sp472 (Type 1E), 7) Lambda ladder.

Group	MRP subtype	*n*	Serotype[a]	*php* RFLP		MIC range (μg/ml)		Resistance Profile
				2b	*2x*	Pen	Cef	
1	1A	91	19F[b] (14)	A	A/B	1-8	0.5-16	SXT, ERY, TET
(n=97)	1B	1	nd	A	A	4	2	SXT, ERY, TET
	1C	1	19[c]	A	B	4	8	SXT, ERY, TET
	1D	1	nd	B	B	2	2	SXT, ERY, TET
	1E	3	nd	A	A	1-4	0.5-1	SXT, ERY, TET
2	2A	9	23F (3)	B	B/A	2-4	1-2	SXT, ERY, CHL[d]
(n=25)	2B	1	23F	B	B	2	1	SXT, ERY, CHL
	2C	1	23F	B	B	2	1	SXT, ERY, CHL
	2D	1	nd	B	B	2	1	SXT, ERY, CHL
	2E	2	23F (1)	B	B	2-4	1-2	SXT, ERY, CHL[d]
	2F	1	23F	B	B	2	1	SXT, ERY, TET, CHL
	2G	3	23F (1)	B	B	2	1-2	SXT, ERY, CHL[d]
	2H	1	23F	B	A	2	2	SXT, ERY, TET, CHL
	2I	1	nd	B	B	4	1	SXT, ERY, TET, CHL
	2J	1	nd	B	B	1	0.5	SXT, TET, CHL
	2K	1	23F	B	B	2	1	SXT, ERY, TET, CHL
	2M	2	nd	G	D/H	0.5-1	0.25-1	SXT, ERY, TET, CHL
	2N	1	nd	B	B	2	1	SXT, TET, CHL
3	3A	6	nd	B	B	1-4	1-2	SXTe
(n=24)	3B	6	14 (1)	B	B	1-4	1-2	SXTe[f]
	3C	4	nd	B	B	0.5-1	0.5-1	SXT
	3D	4	nd	B	B	0.5-1	0.5	SXT
	3E	4	9[c] (1)	B	B	0.5-2	0.5-1	SXTf

Table 3.2 Distribution of MRP subtypes, and their associated serotypes and antibiotic resistance profiles, among the three major groupings of penicillin-resistant pneumococci in Christchurch.
(*a*) Number of isolates serotyped represented in parentheses, (*b*) 14 isolates confirmed as serogroup 19, 4 typed further as serotype 19F, (*c*) Only serogroup determined, (*d*) Some isolates also resistant to tetracycline, (type 2A – 2 resistant, type 2E – 1 resistant, type 2G – 2 resistant), (*e*) not all isolates resistant to co-trimoxazole (type 3A – 4 resistant, type 3B – 3 resistant), (*f*) one isolate resistant to erythromycin and tetracycline but sensitive to co-trimoxazole. *nd* not determined, SXT co-trimoxazole, ERY erythromycin, TET tetracycline, CHL chloramphenicol.

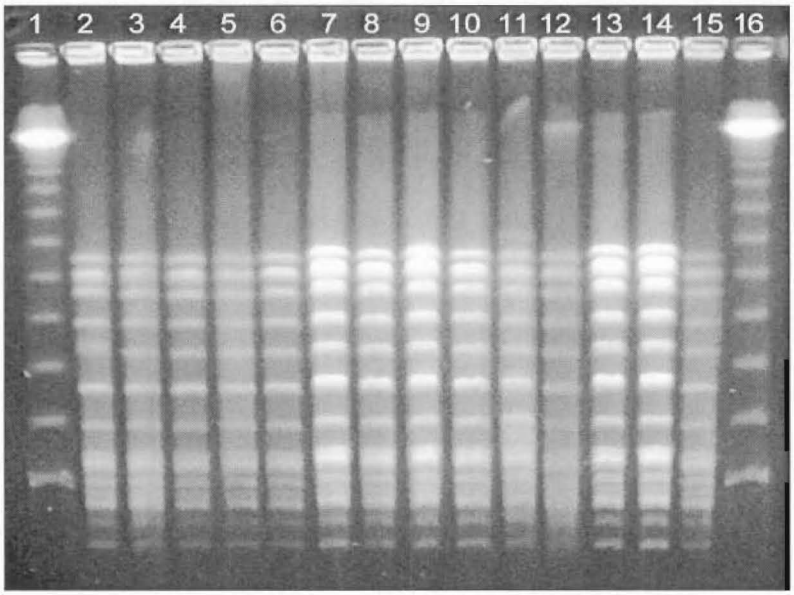

Figure 3.4 *Apa*I genomic digests of isolates belonging to MRP 1A
Lanes 1) Lambda ladder, 2) isolate Sp439, 3) isolate Sp440, 4) isolate Sp443, 5) isolate Sp447, 6) isolate Sp453, 7) isolate Sp456, 8) isolate Sp458, 9) isolate Sp459, 10) isolate Sp461, 11) isolate Sp462, 12) isolate Sp467, 13) isolate Sp473, 14) isolate Sp474, 15) isolate 99-539, 16) Lambda ladder.

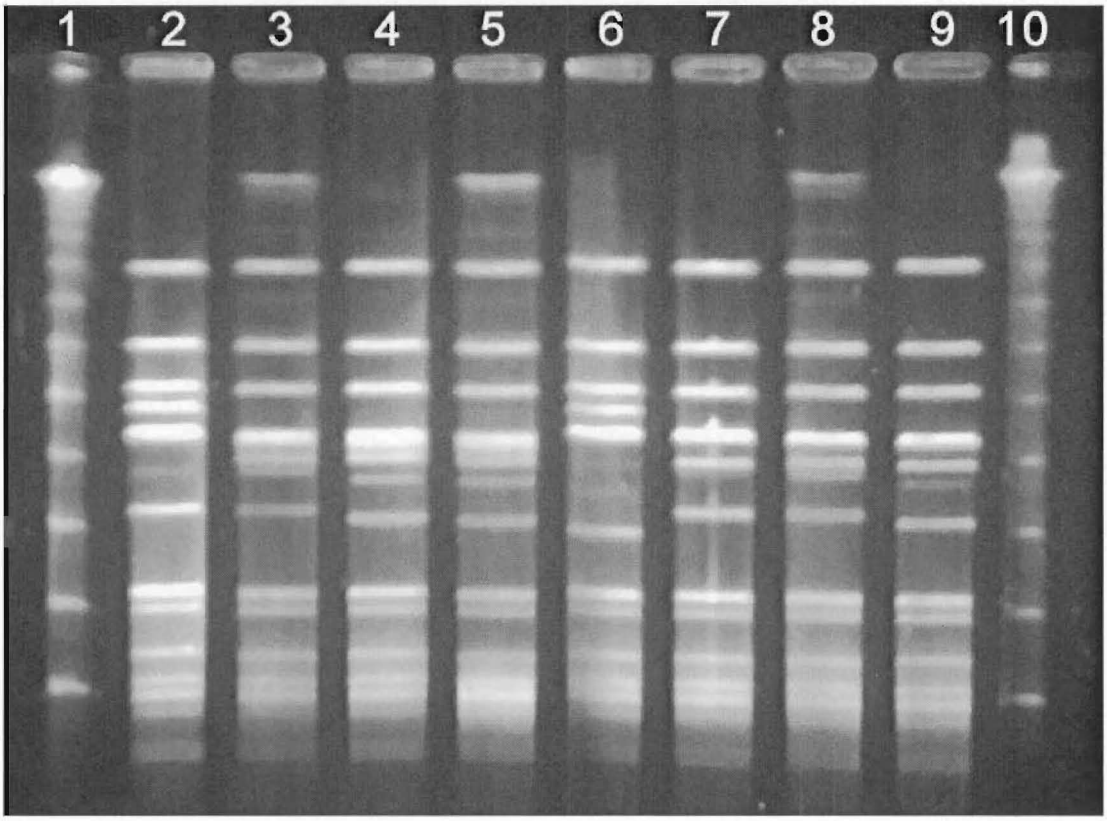

Figure 3.5 Comparison of the *Sma*I DNA macro-restriction subtypes of the major MRP group 2 predominant among Christchurch penicillin non-susceptible isolates of *S. pneumoniae*.
Lanes 1) Lambda ladder, 2) Strain ATCC 700669 (Spain^{23F}-1), 3) isolate Sp020 (Type 2A), 4) isolate Sp001 (Type 2B), 5) isolate Sp008 (Type 2C), 6) isolate Sp014 (Type 2E), 7) isolate Sp004 (Type 2F), 8) isolate Sp045 (Type 2G), 9) isolate Sp030 (Type 2H), 10) Lambda ladder.

Macro-restriction group 2 (Figure 3.2) was the second largest group containing 25 (12.5%) of the isolates. The group was genetically heterogeneous, which is evident by the many subtypes observed associated with this group. These subtypes arose due to slight, but distinct, changes in mobility of individual bands around 200-250 kb (Figure 3.5), presumably the result of insertions or deletions. A total of 13 different subtypes were identified, each containing from one to nine isolates (Table 3.2). Isolates within group 2 had a range of penicillin MICs from 0.5-4.0 μg/ml. All isolates were multi-resistant, typically exhibiting resistance to co-trimoxazole, erythromycin and chloramphenicol, with some isolates also resistant to tetracycline (Table 3.2). Ten representative isolates from this group were serotyped, and expressed capsular type 23F.

Macro-restriction group 3 (Figure 3.2) consisted of 24 isolates in five types. Each subtype contained between four and six isolates (Table 3.2). Penicillin MICs for this group ranged from 0.5-4 μg/ml. This group was typically not multi-drug resistant, although several isolates were resistant to co-trimoxazole as well as penicillin. Only two members of this group were serotyped, and were found to be serotype 9 and 14 (Table 3.2).

Macro-restriction group 11 contained 25 isolates distributed among ten subtypes. Each subtype contained between one and eight isolates. Quite a degree of heterogeneity was exhibited between MRPs. However, most of the diversity observed could be attributed to a single *Sma*I fragment, which varied in size between 100–150 kb (Figure 3.6). Group 11 isolates were all intermediately resistant to penicillin (MICs 0.12-0.25 μg/ml), but all were multi-drug resistant. All group 11 isolates were resistant to co-trimoxazole and erythromycin. Most (96%) were also resistant to tetracycline. Only two group 11 isolates were serotyped, and both of these were found to be untypable (unencapsulated or an unrecognised serotype). The MRP 11 was associated predominantly with eye swabs Table 3.3.

MRP type	Eye	Sputum	Ear	Nose	Other
Type 1 (*n*=97)	18	22	38	12	10
Type 2 (*n*=25)	16	36	24	8	16
Type 3 (*n*=24)	12.5	12.5	33.5	12.5	29
Type 11 (*n*=25)	92	4	0	0	4

Table 3.3 Proportion (%) of MRP types and their association with main body sites.

Macro-restriction profiles 4-10 and 12-25 represented unique profiles that differed significantly from each of the major groups, and from each other. Of these 21 groups, most (81%) contained

only one isolate representative of that profile. Four groups (5, 9, 10 and 12) could be differentiated into two subgroups, the largest of which (9A) contained three isolates.

Group	MRP subtype	*n*	Serotype[a]	*pbp* RFLP		MIC range (μg/ml)		Resistance Profile
				2b	*2x*	Pen	Cef	
11	11A	2	*nd*	D	1	0.12	0.12	SXT, ERY, TET
(*n*=25)	11B	1	*nd*	D	1	0.12	0.12	SXT, ERY, TET
	11C	6	*nd*	D	1	0.12	0.12	SXT, ERY, TET
	11D	8	nt (1)	D	1	0.12	0.12	SXT, ERY, TET
	11E	2	*nd*	D	1	0.12	0.12	SXT, ERY, TET
	11F	1	*nd*	D	1	0.12	0.12	SXT, ERY
	11G	1	*nd*	D	1	0.12	0.12	SXT, ERY, TET
	11H	1	*nd*	D	1	0.25	0.25	SXT, ERY, TET
	11I	1	*nd*	D	1	0.12	0.12	SXT, ERY, TET
	11J	2	nt (1)	D	1	0.12	0.12	SXT, ERY, TET[a]

Table 3.4 Distribution of MRP subtypes, and their associated serotypes and antibiotic resistance profiles, among the group 11 penicillin-resistant pneumococci in Christchurch.
nd not determined, nt non-typable, SXT co-trimoxazole, ERY erythromycin, TET tetracycline.

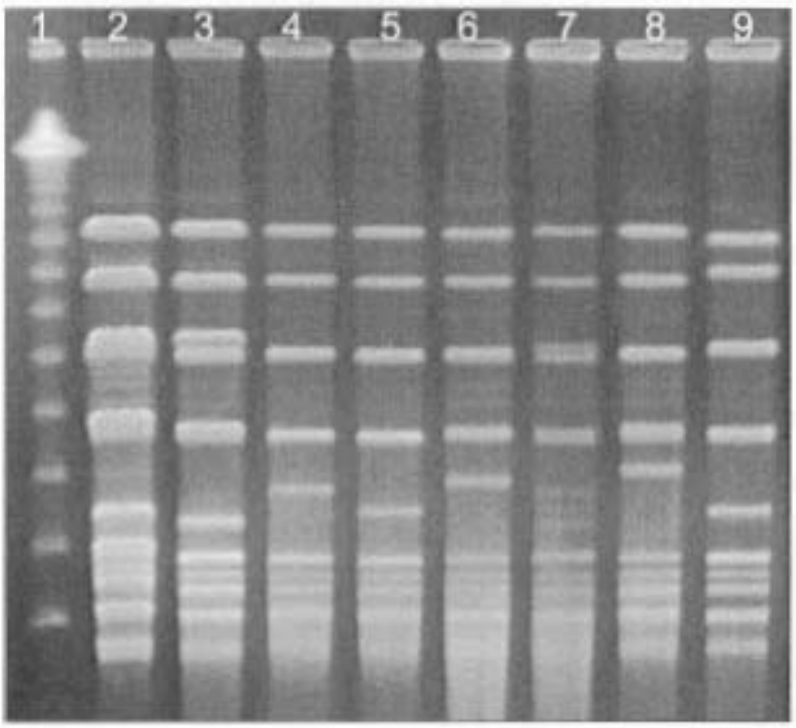

Figure 3.6 Comparison of the *Sma*I DNA MRP subtypes of the group 11 isolates of Christchurch penicillin non-susceptible *S. pneumoniae*.
Lanes 1) Lambda ladder, 2) isolate Sp023 (Type 11A), 3) isolate Sp024 (Type 11B), 4) isolate Sp056 (Type 11C), 5) isolate Sp058 (Type 11D), 6) isolate Sp342 (Type 11E), 7) isolate Sp351 (Type 11F), 8) isolate Sp452 (Type 11G), 9) isolate Sp457 (Type H).

Group	MRP subtype	*n*	Serotype[a]	*pbp* RFLP		MIC range (µg/ml)		Resistance Profile
				2b	*2x*	Pen	Cef	
4	--	1	*nd*	O	I	1	1	ERY, TET
5	5A	1	*nd*	F	I	1	1	SXT
	5B	1	*nd*	L	O	0.5	*nd*	SXT
6	--	1	*nd*	M	E	1	*nd*	
7	--	1	nt	B	B	2	0.5	SXT, TET, CHL
8	--	1	6	B	B	2	1	SXT, ERY, TET, CHL
9	9A	3	19 (2)	G	G	1-2	1	ERY, TET
	9B	1	*nd*	G	G	2	1	SXT, ERY, TET
10	10A	2	19F	B	B	2-4	2-4	SXT, ERY, TET, CHL
	10B	1	19F	B	B	2	2	SXT, ERY, TET, CHL
	10C	1	19F	B	B	2	1	SXT, ERY, TET, CHL
12	12A	1	*nd*	H	J	0.12	0.06	SXT
	12B	1	*nd*	I	L	0.12	0.06	SXT
13	--	1	*nd*	E	B	0.12	0.5	ERY, TET
14	--	1	*nd*	E	L	0.12	0.12	
15	--	1	nt	A	B	2	1	ERY
16	--	1	*nd*	E	L	0.12	0.06	SXT
17	--	1	*nd*	I	L	0.12	0.06	SXT
18	--	1	*nd*	A	B	1	1	SXT, ERY, TET
19	--	1	*nd*	A	A	2	0.5	SXT, ERY, TET
20	--	1	*nd*	B	B	1	*nd*	SXT
21	--	1	*nd*	B	D	0.12	0.12	ERY
22	--	1	*nd*	N	B	1	0.5	SXT, TET, CHL
23	--	1	*nd*	A	L	1	*nd*	SXT, TET
24	--	1	*nd*	I	L	0.12	*nd*	SXT
25	--	1	*nd*	C	B	0.12	0.12	SXT, ERY, TET

Table 3.5 Distribution of *Sma*I MRP subtypes, and their associated serotypes and antibiotic resistance profiles, among the minor groups of penicillin-resistant pneumococci in Christchurch. *nd* not determined, nt non-typable, SXT co-trimoxazole, ERY erythromycin, TET tetracycline, CHL chloramphenicol.

3.2.3 Analysis of *pbp* genes

Gene fragments encompassing the transpeptidase (penicillin binding region) coding region of *pbp2b* and *2x* were amplified using the primers described in Table 2.4. Amplicons were 1.5 kb and 2.0 kb for the *pbp2b* and *2x* genes respectively and subsequent digestions were performed with the enzymes *Dde*I (*pbp2b*) and *Hin*fI (*pbp2x*) (Figure 3.7 and Figure 3.9 respectively). RFLP analysis was performed on both genes for all 200 isolates, and comparison of resulting profiles was performed visually. The association of *pbp* RFLP types with MRPs and their associated β-lactam MICs is shown in Table 3.2 through to 3.4. Graphical interpretations of the RFLP profiles for both *pbp2b* and *2x* are shown in Figure 3.8 and Figure 3.10 respectively. These figures document all diversity observed in the *pbp* RFLP patterns, including that observed in Chapter four.

3.2.3.1 RFLP analysis of the pbp2b *gene*

Among the 200 Christchurch pneumococcal isolates, 13 different RFLP profiles were observed. The three most frequently occurring profiles (A, B and D), associated with 101 (50.5%), 54 (27%) and 25 (12.5%) isolates respectively.

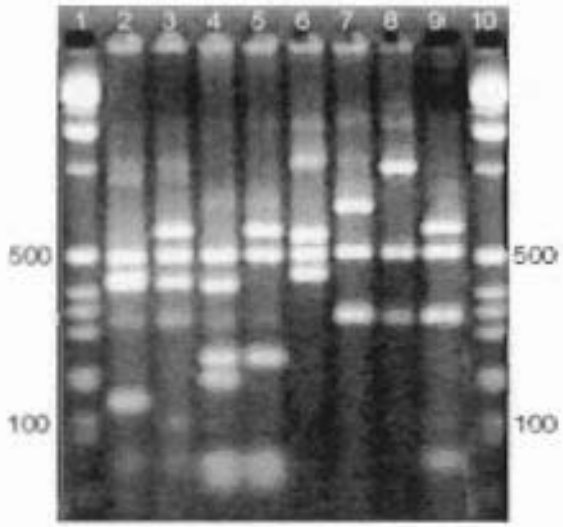

Figure 3.7 Representative *pbp2b* RFLP patterns.
Lanes 1) and 10) 1kb extension ladder, Lane 2) Type A, Lane 3) Type B, Lane 4) Type D, Lane 5) Type E, Lane 6) Type F, Lane 7) Type G, Lane 8) Type K, Lane 9) Type I.

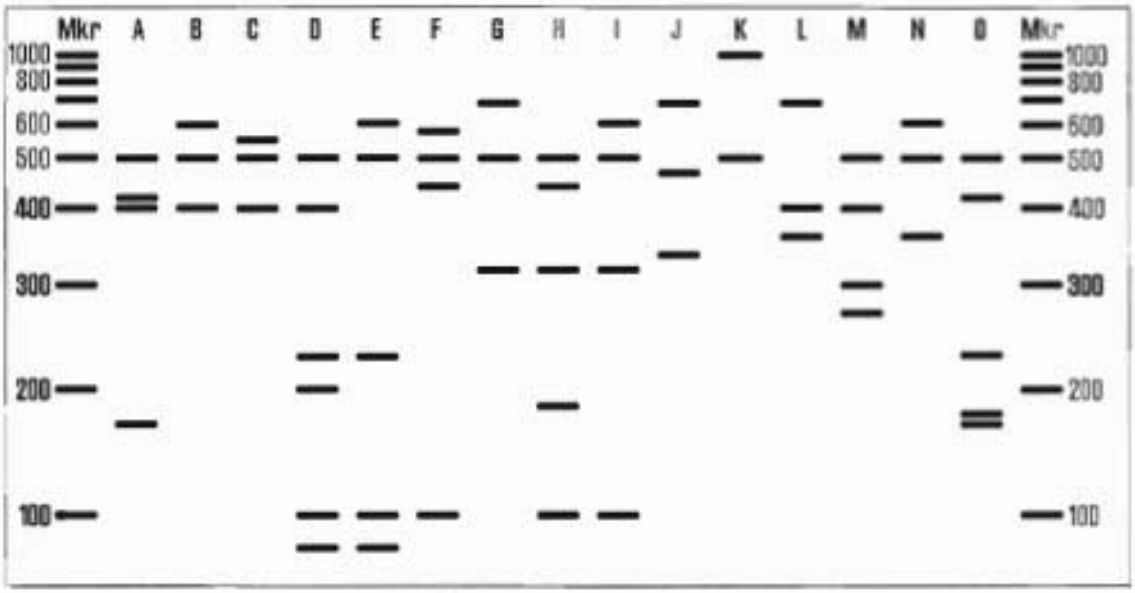

Figure 3.8 Graphical interpretation of all *pbp2b* RFLP profiles observed. This includes profiles of strains from following chapters.
(Marker in bp); Bands smaller than 100 bp have been omitted due to the difficulty resolving small DNA fragments by agarose electrophoresis.

3.2.3.2 RFLP analysis of the pbp2x *gene*

Among the 200 Christchurch pneumococcal isolates, ten different RFLP profiles were observed. The three most frequently occurring profiles (A, B and I), associated with 87 (43.5%), 69 (34.5%) and 26 (13%) isolates respectively.

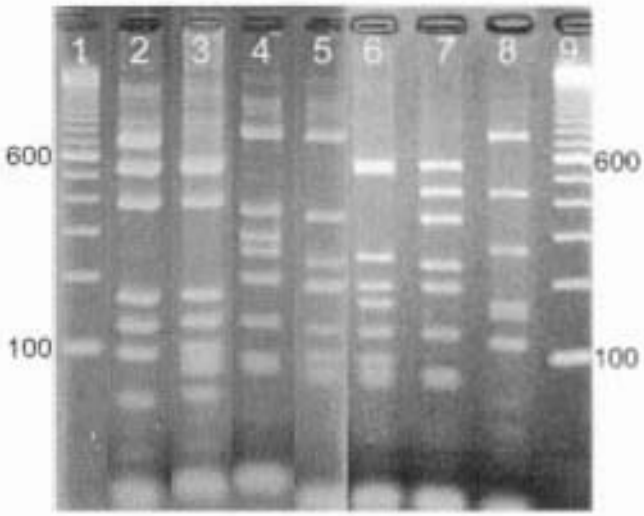

Figure 3.9 Composite image of representative *pbp2x* RFLP patterns.
Lanes 1) and 9) 100 bp ladder, Lane 2) Type A, Lane 3) Type B, Lane 4) Type C, Lane 5) Type D, Lane 6) Type H, Lane 7) Type L, Lane 8) Type M

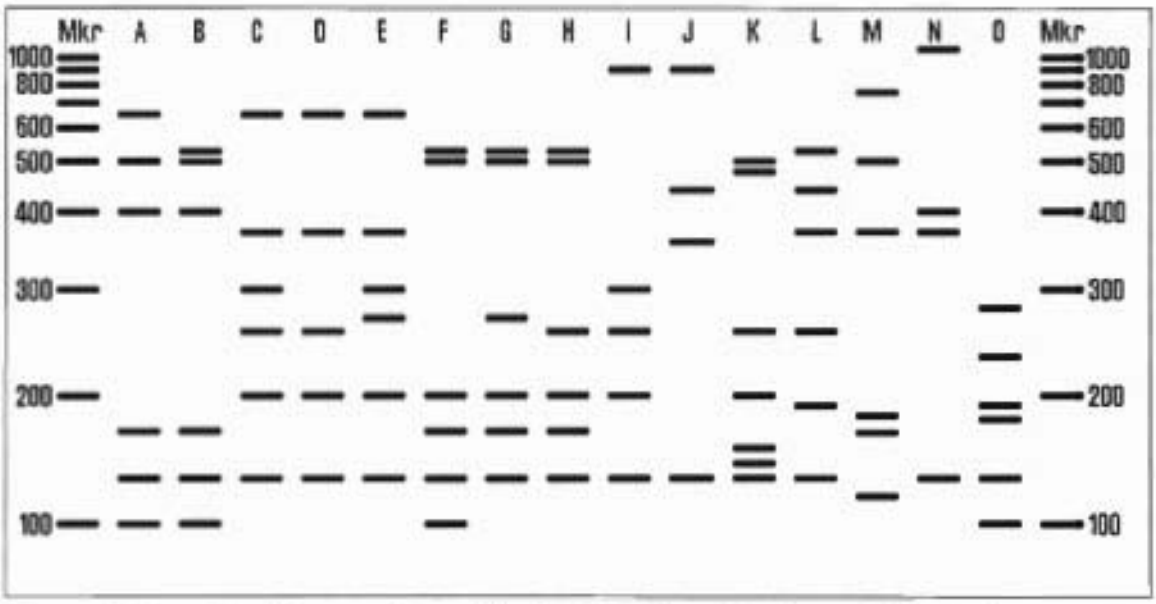

Figure 3.10 Graphical interpretation of all *pbp2x* RFLP profiles observed. This includes profiles of strains from following chapters.
Marker in bp. Bands smaller than 100 bp have been omitted due to the difficulty resolving small DNA fragments by agarose electrophoresis.

3.3 Discussion

The isolation frequency of penicillin non-susceptible pneumococci has increased significantly in many parts of the world during the 1990s. Examination of resistant isolates by conventional phenotypic techniques may reveal a common serotype or antibiotic resistance profile among the isolates, suggesting the clonal spread of an organism with these traits. The application of molecular techniques adds further resolution to such analysis and can effectively provide discrimination between clonally related isolates, and genetically unrelated isolates sharing a common phenotype. When applied to rapidly emerging resistant pneumococcal populations, clonal lineages are often found to comprise a large proportion of the population. Macro-restriction profiling by PFGE is a powerful tool in such analysis.

In the current study, PFGE discriminated 59 distinct MRPs from a total sample of 200 pneumococcal isolates. From this data it was shown that 86.5% of the isolates could be assigned to one of four macro-restriction groups, the largest of which contained 97 (48.5%) of the isolates. Two of the four major PFGE groups (MRPs 2 and 3), could tentatively be described as belonging to globally widespread epidemic clones; the Spain^{23F}-1 clone and the France^{9V}-3 clone respectively. Isolates belonging to the PFGE group 2 were phenotypically (as assessed by serotyping and antibiotic susceptibility - Table 3.2) and genotypically (as assessed by PFGE - Figure 3.5) similar to the Spain^{23F}-1 clone. This clonal group comprised 12.5% of the Christchurch sample of resistant isolates. It is not surprising to find this isolate in New Zealand given its broad international spread.

Besides the two international clones, another two MRP groups contained significant proportions of resistant isolates. PFGE groups 1 and 11 each contained 97 (48.5%) and 25 (12.5%) isolates, respectively. Each of these two groups has similar antibiotic susceptibility profiles; they are both resistant to erythromycin, co-trimoxazole and tetracycline, but sensitive to chloramphenicol. The main distinguishing feature between the two clonal groups is their respective penicillin MICs. Isolates from PFGE group 1 typically have penicillin MICs ranging from 1-8 μg/ml while group 11 tend to be of intermediate penicillin resistance (MIC 0.12 μg/ml).

The PFGE group 1 comprised nearly half the penicillin-resistant isolates from Christchurch included in this study. At the time this work was performed, this represented a hitherto undescribed multi-resistant pneumococcal clone. This observation provided the stimulus for much of the work described in this study, as this clone was characterised in more detail. As will

be shown in following chapters, the predominant New Zealand clone was shown quite convincingly to be a variant of a recently described clone from Taiwan (Shi et al., 1998).

The fourth major MRP group is interesting in that it is multi-drug resistant (erythromycin, co-trimoxazole and tetracycline), yet it has only intermediate resistance to penicillin (MIC 0.12 μg/ml). Unfortunately this clone had few other distinguishing features – the two isolates submitted for serotyping were non-typable. Due to the low level of penicillin resistance in this clone, it was not investigated any further, other than this initial observation. However, the relative abundance of this clone as well as its multi-drug resistant phenotype, make it a good candidate for future investigation.

3.3.1 Intercontinental spread of resistant pneumococci

3.3.1.1 Spread of the Spain^{23F}-1 clone

As discussed in Chapter one, the best-described multi-resistant pneumococcal clone is the Spain^{23F}-1 clone. The intercontinental spread of this clone was first demonstrated in 1991 (Munoz et al., 1991), and has subsequently been reported worldwide. The origin of penicillin resistance in Spain is attributed to the high use of antibiotics in this country; in 1983 Spain prescribed 1.5 times more penicillin than the France, 1.7 times that of Italy, and 5.4 times more than the UK (Baquero et al., 1991). Furthermore, a clear linear relationship was shown to exist in Spain between the amount of penicillin used in a year, and the prevalence of resistance (Baquero et al., 1991).

Given the prolific nature of this pneumococcal clone, it is not possible to speculate on a potential source of introduction into New Zealand. It is quite feasible that this clone has been introduced multiple times, from many locations.

3.3.1.2 Spread of the France^{9V}-3 clone

The France^{9V}-3 clone, like the Spanish 23F clone, has a global distribution and for this reason it is not possible to speculate an immediate source of this clone in New Zealand. Studies on members of this clone have demonstrated its capacity to disseminate globally, and establish itself in regions geographically widespread (assuming France to be the origin of this clone). For example, a study in Uruguay documented the spread of an increase penicillin and co-trimoxazole resistance within its paediatric population (Camou et al., 1998). The use of PFGE identified a common MRP among 77% of the isolates, which was found to be indistinguishable from that of the France^{9V}-3 clone. The predominant serotype associated with this clone in

Uruguay was 14, suggesting that serotype switching had occurred in this clone, this was later confirmed by Coffey and colleagues (1999). The serotype 14 France^{9V}-3 clone was first detected in 1992, and rapidly became the dominant resistant clone (Camou et al., 1998).

Like the Spain^{23F}-1 clone, an origin of importation cannot be determined for the France^{9V}-3 clone. Both clones are well-established throughout the globe and could have effectively been introduced from anywhere. This study shows they are also present and established in New Zealand and contribute significantly to the resistance burden.

3.3.1.3 Origin of the major New Zealand clone

In 1997, 228 pneumococcal isolates with high level penicillin resistance (MIC ≥ 2 μg/ml) were submitted to ESR, Kenepuru. Of these, 114 (50%) belonged to serogroup 19 (46 of which were further serotyped as 19F) (Brett, 1997). Significantly, 1997 marked a large increase in prevalence of pneumococcal resistance to cefotaxime (MIC ≥ 2 μg/ml), which increased from 5.4% in 1996 to 36.6% in 1997. Notably, the cefotaxime-resistant isolates included 30 with very high resistance (MIC 8-32 μg/ml). These isolates were all serogroup 19 and referred mainly from Christchurch and Palmerston North (Brett, 1997). DNA MRP analysis of these strains also suggested that these isolates were clonal (Brett, 1997, Brett, 2001). This was the first report of the epidemic multi-resistant 19F clone that is now prolific in New Zealand; in the following chapter it will be shown that this clone was prevalent in New Zealand as early as 1995.

At the time, no descriptions of a multi-resistant 19F pneumococcal clone which matched the New Zealand clone existed in the literature. This suggested that the cefotaxime-resistant pneumococcal clone might have been an indigenous clone; however, the literature suggested that the rapid establishment and spread of a resistant clone is generally due to the importation of a pre-existing clone. This observation was a major impetus for this study.

This study shows that the New Zealand 19F clone is genetically very homogenous as assessed by MRP analysis. This suggests that this novel clone may have only recently begun to disseminate. Other globally widespread pneumococcal clones have small, but significant changes in their MRP profiles from evolutionary divergence of individual lineages. It was realised that this homogeneity also provided a unique opportunity to study the cefotaxime-resistance mechanism in these isolates. Although there was some variation observed in the cefotaxime MICs, the MRP profiles suggested the genetic backgrounds were comparable between isolates.

Reference : Portions of this chapter have been published in *Streptococci and Streptococcal Diseases – Entering the new Millennium*, Proceedings of the XIV Lancefield Symposium on Streptococci and Streptococcal Disease, 2000. pp 229-234. Edited by Martin, D. R. and Tagg, J. R. Securacopy. New Zealand.

Chapter 4

Global Epidemiology

4.1 Introduction

Microorganisms do not respect the artificial constraints of national borders. As illustrated in previous chapters, many clonal strains of *S. pneumoniae* have been shown to have successfully spread throughout the world (Munoz et al., 1991, McGee et al., 1997). In such instances, molecular methods such as macro-restriction profiling (MRP) have been used to show that isolates from one country were indistinguishable, or closely related to, those from another country. Analyses such as these are useful, particularly when a geographical or temporal epidemiological link may provide insight as to the origin of a given bacterial isolate. However, these links are not necessarily immediately obvious. In the previous chapter, an undescribed, multi-resistant pneumococcal clone was shown to be the predominant clone among New Zealand isolates. Two options exist with regard to the origin of this clone; 1) this clone arose in New Zealand, or 2) this clone evolved elsewhere in the world and was subsequently imported into New Zealand. Numerous reports have been published in the last 20 years documenting movement of resistant pneumococcal clones between different countries, and it was, therefore, hypothesized that this would be the case in New Zealand. However, New Zealand's geographic isolation coupled with high levels of tourism and migration into the country, mean the potential source of the resistant pneumococcal clone is ambiguous; the clone could literally have arisen anywhere in the world. In order to investigate the origin of this clone, a methodology for global epidemiology is necessary.

To track the movement of microorganisms on a global scale requires a change in strategy from that of local epidemiology. The molecular methods described in previous chapters (e.g., PFGE and PCR based identification) are based on genomic variations between isolates of a given species. These methods are designed to exploit variation between genomes in such a way that maximal discrimination between unrelated isolates will be observed. This is achieved by using multiple target sequences throughout the genome, whether they are restriction sites, or repetitive PCR primer binding sites. Any significant variation that occurs within the genome is likely to affect the relative distribution of such targets, witnessed by an effect on the profile obtained. By

way of example, should a transposon containing one new restriction site integrate into the genome of a given isolate, it will result in a difference of up to three bands when compared by MRP to the original organism (Tenover et al., 1995, Thal et al., 1997). In such an instance the two isolates would still have homogenous genetic backgrounds (with the exception of the transposon), yet their respective MRP patterns would be dissimilar (Thal et al., 1997). In such a way, relationships between isolates can become rapidly blurred. In short, such methods are designed to target DNA sequence variation that tends to accumulate relatively rapidly to maximise the possibility that any significant changes in genotype of isolates will be detected. The rapid acquisition and ease of detection of this variation provides these methods with a high level of discriminatory power. Consequently they are very useful for short-term epidemiological studies or outbreak investigation (Tenover et al., 1995).

However, for longer-term epidemiological investigations, methodologies which index rapidly acquired genetic variation can be less than satisfactory (Enright and Spratt, 1999b). For example, when comparing isolates from distant geographic locations, strains may have arisen from a common ancestor but rapid acquisition of genetic variation could well mask this relationship. To embark on long-term epidemiological studies, markers that provide a high degree of discrimination between isolates are still required, however the variation between these markers must accumulate at a rate slow enough not to provide a long-term clonal stability. This can be achieved by using selectively neutral targets, such as housekeeping genes. This works on the assumption that housekeeping genes are not under the influence of external selective pressures and consequently any variation observed between isolates is likely to be the cause of random rearrangements (whether these arise through point mutations, insertions, or recombination is academic).

A method exploiting the slow accumulation of variation in housekeeping genes, is multilocus enzyme electrophoresis (MLEE). MLEE is a technique that compares the relative electrophoretic mobilites of housekeeping enzymes on starch gels (Selander et al., 1986). Variation in the primary DNA sequence of genes may lead to the incorporation of different amino acids into enzymes. This in turn may alter the overall charge on an enzyme, which can be observed as an altered mobility when the enzyme is subjected to electrophoresis. To overcome the limited variability that might be expected at any one loci, MLEE utilizes multiple enzymes as targets to maximise variation. Up to twenty-five different loci may be examined for maximum resolving power (Selander et al., 1986). Although MLEE has many attractive features

as a system for strain differentiation, it suffers from being laborious, somewhat subjective and the results generated are difficult to compare between laboratories (Enright and Spratt, 1999b).

A technique that builds on the principles of MLEE – multilocus sequence typing (MLST) – has recently been described (Maiden et al., 1998, Enright and Spratt, 1999b, Spratt, 1999). Multilocus sequence typing also documents slowly acquired variation in the housekeeping enzymes, but is based on the partial DNA sequence (~500 bp) of the genes encoding these enzymes rather than the electrophoretic mobilities of the enzymes themselves. As such each individual nucleotide may be interpreted as a unique character in a comparison between isolates. This gives the MLST system several benefits over MLEE; (1) fewer loci need be examined as MLST documents all nucleotide changes, including silent mutations which may not alter the amino acid composition of any given enzyme; (2) sequence data is unambiguous, whereas banding patterns on electrophoretic gels are subject to user interpretation, and; (3) sequence data is electronically portable allowing rapid comparison and sharing between laboratories via the internet.

MLST was initially validated on a set of 107 isolates of *N. meningitidis* isolated from invasive disease that had previously been studied by MLEE (Maiden et al., 1998). Eleven housekeeping genes were selected initially and their partial nucleotide sequence (~450bp) were determined. The allelic profiles of the eleven loci resolved 74 sequence types among the 107 strains. The results obtained from MLST were highly congruent with those obtained by MLEE, with a few exceptions (Maiden et al., 1998). As the congruence between sequence data and MLEE was better for some gene fragments than others, a subset of six loci was chosen that produced a dendrogram which correlated almost perfectly with that expected from the MLEE data (Maiden et al., 1998). The first application of MLST to clinical isolates of *N. meningitidis* included the addition of a seventh locus (Feavers et al., 1999) to provide greater resolution between lineages. MLST schemes have been subsequently extended to cover several other bacterial species including *Streptococcus pneumoniae* (Enright and Spratt, 1998), *Campylobacter jejuni* (Dingle et al., 2001), *Staphylococcus aureus* (Enright et al., 2000), and *Streptococcus pyogenes* (Enright et al., 2001). At the time of this writing, MLST databases were also being compiled for *Streptococcus agalactiae*, *Haemophilus influenzae* and *Burkholderia pseuodomallei*.

In practice, application of MLST to a pneumococcal population first requires isolation of chromosomal DNA from the isolate of interest; PCR amplification of internal gene fragments of several housekeeping genes; and determination of the nucleotide sequence of the gene

fragments on both strands, using the same primers as the initial PCR (Spratt, 1999). Once obtained the forward and reverse sequences are aligned for comparison, ambiguities resolved and trimmed to the length specified for the given gene fragment. To determine if a gene sequence has been observed before, the MLST database may be queried via the Internet (http://www.mlst.net). Every allele of each loci within the database has been assigned an arbitrary number. The sequence determination at all seven loci (*aroE, gdh, gki, recP, spi, xpt* and *ddl*; see Table 2.3 for complete descriptions) provides an allelic profile (e.g., the allelic profile of the major Spain^{23F}-1 multi-resistant clone is 4, 4, 2, 4, 4, 1, 1). The allelic profile of an isolate defines the given isolate's sequence type (ST). Designation of a ST may be performed via an interrogative interface on the MLST website.

Typically pneumococcal isolates are considered to be members of the same clonal complex if they have the same allelic profile or differ at one loci. Phylogenetic relationships between isolates that differ at more than three of the seven loci are likely to be unreliable (Enright and Spratt, 1999b). For the purpose of this work, pneumococcal isolates are organised into clusters according to major clones defined by the pneumococcal molecular epidemiology network (PMEN) when possible (see Table 1.4 for descriptions).

4.2 Results

A total of 76 isolates of *S. pneumoniae* were chosen for MLST (Appendix A.I.ii and A.I.iii). Of these, 74 isolates were obtained from throughout New Zealand between 1992 and 2000, and two representative PMEN clones were included as internal controls. The control strains used were ATCC 700671, (Spain^{9V}-3) and ATCC 700669 (Spain^{23F}-1). Pneumococcal isolates were selected for characterisation by MLST to fulfil three criteria. Firstly, typical isolates of each of the major clones in New Zealand (as determined by PFGE and serotyping) were characterised; secondly, isolates were selected retrospectively to establish when major clones were first recovered in New Zealand; thirdly, isolates with novel phenotypes (e.g., antibiotic resistance uncharacteristic of a given clone) or genotypes (e.g., members of a clonal group with an uncharacteristic PFGE profile) were.

Isolates were selected from hospitals and laboratories throughout New Zealand (Figure 4.1) with 47 (63.5%) included from the North Island and 27 (36.5%) from the South Island. The two major contributing centres were Auckland and Christchurch, with 25 (33.8%) and 22 (29.7%) isolates from each, respectively.

Of the 74 NZ strains 50 (67.6%) were strains from invasive disease. The remaining 24 (32.4%) strains were isolated from non-invasive sites and were included due to their decreased susceptibility to penicillin. There was an uneven distribution of the strains by serotype; 2 serotype 6B (2.7%), 2 serotype 9N (2.7%), 9 serotype 9V (12.2%), 11 serotype 14 (14.9%), 4 serotype 19A (5.4%), 33 serotype 19F (44.6%) and 13 serotype 23F (17.5%) were selected. The disproportionately high number of serotype 19F isolates was due to the high recovery of 19F isolates expressing decreased antibiotic susceptibility and their clonal spread as shown by MRP (Chapter 3).

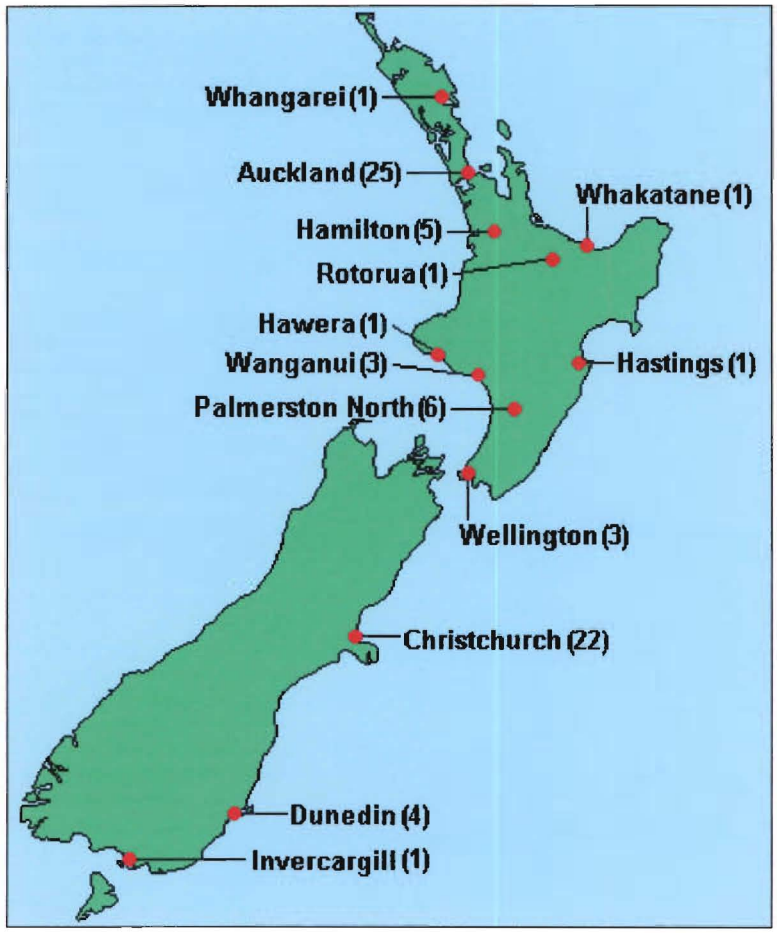

Figure 4.1 Geographic distribution throughout New Zealand of cities from which pneumococcal isolates characterised by MLST were isolated. Number of isolates included from each centre shown in parenthesis.

Fifty-two of the isolates were resistant to penicillin (MIC ≥ 2.0 μg/ml), and seven were of intermediate resistance (MIC 0.12-1.0 μg/ml). The remaining 15 isolates were susceptible to penicillin, and were included due to their involvement in invasive disease. Erythromycin- and chloramphenicol-resistance was encountered in 42 (56.8%) and 19 (25.7%) isolates respectively. Testing for susceptibility to co-trimoxazole and tetracycline was not routinely

performed on invasive isolates from 1997, consequently susceptibility data is available for only 63 and 56 isolates respectively. The observed rate of resistance among isolates included in this study was 60.3% for co-trimoxazole, and 53.6% for tetracycline.

Of the 74 pneumococcal isolates analysed by MLST, 58 (78%) isolates matched previously described pneumococcal STs in the MLST database. The frequency of known STs identified amongst the New Zealand isolates is shown in Table 4.1. The remaining 14 isolates were comprised of allelic profiles for which no ST had been designated. This includes variants of existing STs and completely unique allelic profiles.

ST	*n*	Serotype	PMEN designation	Pen	Cef	Allelic profiles						
						aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
9*	1	14	England^{14}-9	S	S	1	5	4	5	5	1	8
36	2	23F		S/R	S/I	1	8	4	1	1	4	6
66	2	9N		S	S	2	8	2	4	6	1	1
81*	13	14/19F/23F	Spain^{23F}-1	R	I/R	4	4	2	4	4	1	1
83*	1	23F	Spain^{23F}-1	R	I	4	4	2	4	**6**	1	1
90*	2	6B	Spain^{6B}-2	R	I	5	6	1	2	6	3	4
124	2	14		S	S	7	5	1	8	14	11	14
129	1	14		S	S	7	5	1	8	14	11	5
146	1	19F		S	S	7	6	1	2	6	15	14
156*	8	9V/14	France^{9V}-3	R	S/I	7	11	10	1	6	8	1
162*	3	9V	France^{9V}-3	S	S	7	11	10	1	6	8	**14**
199	2	19A		I	S	8	13	14	4	17	4	**14**
236*	5	19F	Taiwan^{19F}-14	R	S/I	15	16	19	15	6	20	26
237*	1	19F	Taiwan^{19F}-14	I	R	15	16	19	15	6	20	**1**
271*	13	14/19F	Taiwan^{19F}-14	R	I/R	**4**	16	19	15	6	20	26
346	1	14		I	S	10	41	47	16	6	14	2

Table 4.1 Distribution of MLST sequence types for 58 New Zealand isolates matching pre-existing allelic profiles in the database.

STs marked with asterisks are those representing PMEN clones or their derivatives. Pen, penicillin; Cef, cefotaxime; Susceptibilities inferred from MICs; S, susceptible (< 0.1 μg/ml); I, intermediate resistance (0.1-1 μg/ml); R, resistant (≥ 2.0 μg/ml)

4.2.1 MLST data by PMEN clonal group

To facilitate analysis of the data obtained from MLST, it is convenient to focus on the major international clones that have been identified in this study. In particular, the clones, as defined by PMEN (Table 1.4), serve as useful standards against which to compare other isolates. The clones of particular interest are Spain^{23F}-1, Spain^{6B}-2, France^{9V}-3 and Taiwan^{19F}-14. Isolates that

do not belong to the PMEN clones will be considered based on their closest match in the MLST database (Section 4.2.2). Isolates that did not closely match (>3 alleles mismatched) any of the entries in the database will be considered separately in section 4.2.3.

4.2.1.1 Spain^{23F}-1 clonal group

Fourteen Spain^{23F}-1 clones were identified (Table 4.2); all but one of these had the allelic profile 4, 4, 2, 4, 4, 1, 1 (ST 81). Strain 97-350 had an altered *spi* allele giving it the profile 4, 4, 2, 4, 6, 1, 1 (ST 83). Ten of the 14 isolates expressed the serotype 23F, an additional three were serotype 19F and one was serotype 14. The first member of the Spain^{23F}-1 clonal group identified in this study was isolated in Auckland during early 1994. Serotype 23F isolates recovered previous to this yielded unrelated allelic profiles suggesting that the Spain^{23F}-1 clone may have arrived in New Zealand around 1993-94.

Strain[a]	Allelic profile							ST	MRP[b]	Sero	MIC[c]		RP[d]	*pbp*[e]	
	aro	*gdh*	*gki*	*rec*	*spi*	*xpt*	*ddl*				P	C		*2b*	*2x*
SP264	4	4	2	4	4	1	1	81	2	23F	2	0.5	S, T, C	B	B
94-008*	4	4	2	4	4	1	1	81	2	23F	2	1	T, C	B	B
94-099*	4	4	2	4	4	1	1	81	2	23F	2	1	S, T, C	B	B
94-212*	4	4	2	4	4	1	1	81	2	23F	2	1	E, S, C	B	B
96-165*	4	4	2	4	4	1	1	81	2	19F	2	1	E, S, T, C	B	B
97-036*	4	4	2	4	4	1	1	81	2	14	2	1	E, S, T, C	B	B
97-350*	4	4	2	4	**6**	1	1	83	2	23F	2	1	E, S, C[f]	B	B
97-550	4	4	2	4	4	1	1	81	2	23F	2	1	E, S, C	B	B
98-122*	4	4	2	4	4	1	1	81	2	23F	2	1	C[g]	B	B
98-136*	4	4	2	4	4	1	1	81	2	23F	2	1	C[g]	B	B
98-283*	4	4	2	4	4	1	1	81	2	19F	4	2	E, C[g]	B	B
98-299*	4	4	2	4	4	1	1	81	2	23F	2	1	E, C[g]	B	B
98-475*	4	4	2	4	4	1	1	81	2	23F	2	1	E, C[g]	B	B
99-074*	4	4	2	4	4	1	1	81	2	23F	2	4	E, C[g]	B	B
00-058	4	4	2	4	4	1	1	81	2	19F	4	8	E, S, T, C	B	B

Table 4.2 Allelic profiles, serotype and antibiotic resistance profiles of 14 clinical isolates and control (SP264/ATCC700669) related to the Spain^{23F}-1 clone (ST 81).

Bold indicates variant allele, (*a*) Isolates marked with asterisk from invasive disease, (*b*) MRP based on profiles described in chapter 3, in not all instances could isolates be assigned to specific groups (*c*) Penicillin, and cefotaxime (respectively) MICs in μg/ml, (*d*) Resistance profile, all isolates tested against erythromycin (E), co-trimoxazole (S), tetracycline (T) and chloramphenicol (C) unless stated otherwise, (*e*) RFLP pattern of PCR amplified *pbp* gene, based on profiles described in chapter 3, (*f*) Resistance to tetracycline not determined, (*g*) Resistance to co-trimoxazole and tetracycline not determined.

Within the Spain^{23F}-1 clonal lineage, *pbp2b* and *2x* RFLP profiles were conserved, as were the corresponding MICs to both penicillin and cefotaxime. A notable exception can be seen, however, in the members of the Spain^{23F}-1 clonal group expressing the 19F capsular type. In two of the three 19F isolates, the MIC is at least one doubling dilution higher for both β-lactams than those isolates expressing the 23F capsule. This suggests that determinants for β-lactam resistance may have been transferred in addition to the genes for synthesis of the type 19F capsule.

4.2.1.2 Spain^{6B}-2 clonal group

Two serotype 6B isolates were included (P93-241 and P96-466), isolated in 1993 and 1996 respectively (Table 4.3). Both isolates were penicillin-resistant (MIC 2.0 μg/ml) and of intermediate cefotaxime-resistance (MIC 1.0 μg/ml). Although sharing similar susceptibilities to β-lactams, they have quite different RFLP patterns for both *pbp2b* and *2x* genes. The two isolates had similar, although not identical PFGE profiles (Figure 4.3). Both were ST 90, the ST associated with the globally widespread multi-resistant Spanish serotype 6B clone (Spain^{6B}-2). These two members of the Spain^{6B}-2 clonal group were also resistant to every other class of antibiotic tested, with the exception of vancomycin. This is entirely consistent with the PMEN description of this multi-resistant clone. Isolate 93-241 was the first reported penicillin-resistant pneumococcus recovered from invasive disease in New Zealand (1993).

Strain[a]	Allelic profile							ST	Sero	MIC[b]		RP[c]	*pbp*[d]	
	aro	*gdh*	*gki*	*rec*	*spi*	*xpt*	*ddl*			P	C		*2b*	*2x*
93-241*	5	6	1	2	6	3	4	90	6B	2	1	E, S, T, C	B	G
96-466*	5	6	1	2	6	3	4	90	6B	2	1	E, S, T, C	G	B

Table 4.3 Allelic profiles, serotype and antibiotic resistance profiles of two clinical isolates related to the Spain^{6B}-2 clone.
(*a*) Isolates marked with asterisk from invasive disease, (*b*) Penicillin, and cefotaxime (respectively) MICs in μg/ml, (*c*) Resistance profile, all isolates tested against erythromycin (E), co-trimoxazole (S), tetracycline (T) and chloramphenicol (C) unless stated otherwise, (*d*) RFLP pattern of PCR amplified *pbp* gene, based on profiles described in chapter 3.

4.2.1.3 France^{9V}-3 clonal group

Eleven pneumococcal isolates were found to belong to the France^{9V}-3 clonal group (Table 4.4). Of these the most common allelic profile was 7, 11, 10, 1, 6, 8, 1 being observed in eight isolates. All isolates with this profile were isolated during 1995 or thereafter, and exhibited reduced susceptibility to β-lactams. Three isolates were included that were susceptible to penicillin, and were all isolated during, or prior to, 1995. Each of these three sensitive strains

had alternative *ddl* alleles, giving an overall allelic profile 7, 11, 10, 1, 6, 8, 14. The acquisition of β-lactam resistance is also coupled with a change in the *pbp* RFLP patterns. Furthermore it should be noted that the *pbp* RFLP profiles are conserved between members of the France^{9V}-3 clonal group and the Spain^{23F}-1 clonal group. In both groups of isolates, these profiles appear to be associated with penicillin resistance, and intermediate cefotaxime-resistance. None of the New Zealand examples of the France^{9V}-3 were found to be multi-drug resistant, although isolate 93-347 did express resistance to co-trimoxazole as well as penicillin. This phenotype is consistent with the PMEN description of this clone. The relatedness of this group is further demonstrated by the similarities between MRPs of members of this clonal group (Figure 4.2).

Strain[a]	Allelic profile							ST	MRP[b]	Sero	MIC[c]		RP[d]	*pbp*[e]	
	aro	*gdh*	*gki*	*rec*	*spi*	*xpt*	*ddl*				P	C		*2b*	*2x*
TL7/1993	7	11	10	1	6	8	1	156	3B	9V	2	1		B	B
94-015*	7	11	10	1	6	8	**14**	162	3B	9V	0.03	0.016	--	D	D
94-115*	7	11	10	1	6	8	**14**	162	3B	9V	0.016	0.03	--	D	D
95-213*	7	11	10	1	6	8	**14**	162	3B	9V	0.016	0.016	--	D	D
95-347*	7	11	10	1	6	8	1	156	3	9V	2	1	S	B	B
97-377*	7	11	10	1	6	8	1	156	3	9V	2	1	--[f]	B	B
97-552*	7	11	10	1	6	8	1	156	3B	9V	2	1	--[f]	B	B
98-206*	7	11	10	1	6	8	1	156	3B	9V	2	1	--[g]	B	B
98-263*	7	11	10	1	6	8	1	156	3	9V	2	0.5	--[g]	B	B
98-286*	7	11	10	1	6	8	1	156	3B	14	2	1	--[g]	B	B
98-356*	7	11	10	1	6	8	1	156	3	9V	2	1	--[g]	B	B
98-555*	7	11	10	1	6	8	1	156	3	14	2	1	--[g]	B	B

Table 4.4 Allelic profiles, serotype and antibiotic resistance profiles of eleven clinical isolates and control (700671/ATCC70067) related to the France^{9V}-3 clone.

Bold indicates variant allele, (*a*) Isolates marked with asterisk from invasive disease, (*b*) MRP based on profiles described in chapter 3, in not all instances could isolates be assigned to specific groups, (*c*) Penicillin, and cefotaxime (respectively) MICs in μg/ml, (*d*) Resistance profile, all isolates tested against erythromycin (E), co-trimoxazole (S), tetracycline (T) and chloramphenicol (C) unless stated otherwise (dashes indicate sensitivity to all four agents), (*e*) RFLP pattern of PCR amplified *pbp* gene, based on profiles described in chapter 3, (*f*) Resistance to tetracycline not determined, (*g*) Resistance to co-trimoxazole and tetracycline not determined.

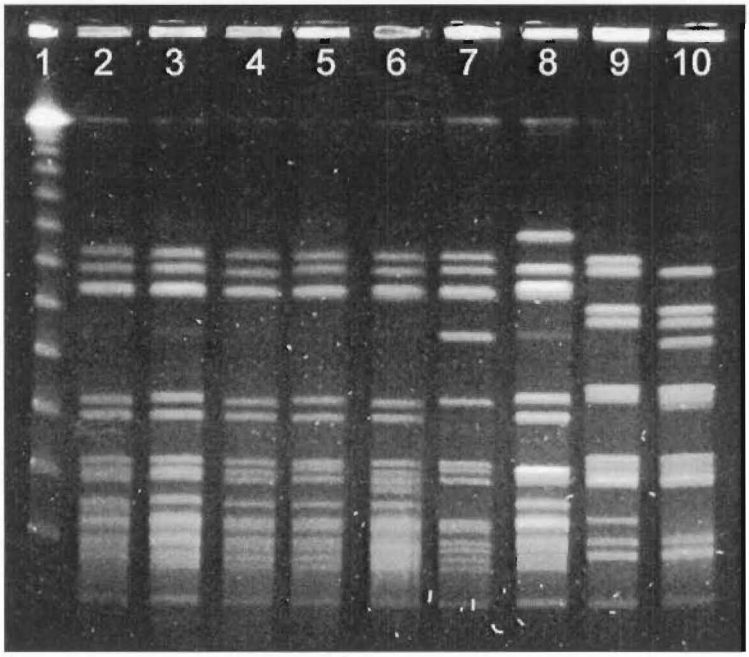

Figure 4.2 DNA macro-restriction profiles of representative isolates expressing serotypes 9V and 9N (including control strain ATCC 700671).
Lanes 1) Lambda ladder, 2) strain ATCC 700671 (ST 156, serotype 9V), 3) P94-115 (ST 162, serotype 9V), 4) P95-213 (ST 162, serotype 9V), 5) P97-552 (ST 156, serotype 9V), 6) P95-347 (ST 156, serotype 9V), 7) P97-377 (ST 156, serotype 9V), 8) P94-015 (ST 162, serotype 9V), 9) P95-298 (ST 66, serotype 9N), 10) P93-075 (ST 66, serotype 9N).

4.2.1.4 England[14]-9 clonal group

One out of the eleven serotype 14 isolates belonged to the PMEN clonal group England[14]-9 (Table 4.5). This clonal group, as described by PMEN, is sensitive to penicillin, but exhibits resistance to erythromycin. Isolate 92-095 corresponds to these phenotypes. Out the five penicillin-susceptible serotype 14 isolates included in this study, 92-095 was the first isolate (in 1992), and the only example of the England[14]-9 clone. This suggests that although the England[14]-9 clone is encountered frequently in Europe, it does not appear to be a problem in New Zealand.

Strain[a]	Allelic profile							ST	Sero	MIC[b]		RP[c]	*pbp*[d]	
	aro	*gdh*	*gki*	*rec*	*spi*	*xpt*	*ddl*			P	C		*2b*	*2x*
92-095*	1	5	4	5	5	1	8	9	14	0.016	0.016	E	nd	C

Table 4.5 Allelic profile, serotype and antibiotic resistance profile of clinical isolate 92-095 - related to the England[14]-9 clone.
(*a*) Isolates marked with asterisk from invasive disease, (*b*) Penicillin, and cefotaxime (respectively) MICs in μg/ml, (*c*) Resistance profile, all isolates tested against erythromycin (E), co-trimoxazole (S), tetracycline (T) and chloramphenicol (C) unless stated otherwise, (*d*) RFLP pattern of PCR amplified *php* gene, based on profiles described in chapter 3.

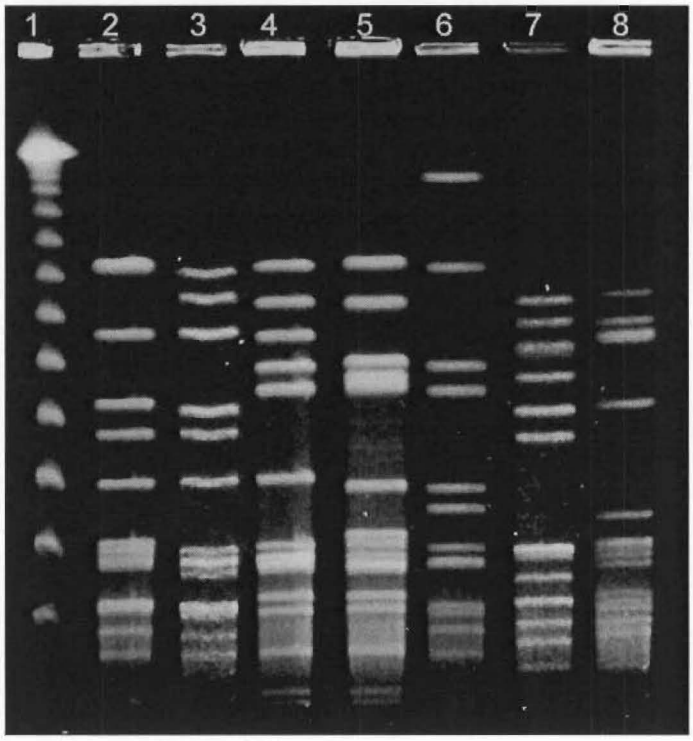

Figure 4.3 DNA macro-restriction profiles of representative isolates expressing serotypes 6B and 14

Lanes 1) Lambda ladder, 2) P93-241 (ST 90, serotype 6B), 3) P96-466 (ST 90, serotype 6B), 4) P95-194 (ST 124, serotype 14), 5) P94-055 (ST 124, serotype 14), 6) P94-001 (ST 129, serotype 14), 7) P92-095 (ST 9, serotype 14), 8) P94-271 (ST undesignated, serotype 14).

Although a RFLP pattern for the *pbp2b* gene of isolate 92-095 was not determined, the *pbp2x* pattern observed is consistent with penicillin susceptibility. The serotype 14 isolates included in this study appear to have considerable genetic heterogeneity as assessed by DNA macro-restriction profiles (Figure 4.3). To this end it is not surprising that ST 9 was observed only once among the New Zealand serotype 14 isolates characterised by MLST.

4.2.1.5 Taiwan^{19F}-14 clonal group

Of the 74 isolates that were characterised by MLST, 26 (35%) belonged to the Taiwan^{19F}-14 clonal group (Table 4.6). All 26 isolates were serotype 19F with the exception of one (99-909) that was type 14. The majority of the New Zealand isolates belonging to this group were actually single locus variants of the Taiwan^{19F}-14 clone, and rather are members of major Korean 19F clone. Of the 26 isolates in this group 14 (54%) had the allelic profile 4, 16, 19, 15, 6, 20, 26 (ST 236) characteristic of the major Korean 19F clone. Five of the 26 (19%) had the allelic profile characteristic of the Taiwan^{19F}-14 clone; 15, 16, 19, 15, 6, 20, 26 (ST 271).

For convenience and ease of explanation, a change in nomenclature is warranted. In breaking from the guidelines established by PMEN (McGee et al., 2001b), the major clone in New

Zealand shall be referred to as the Korea^{19F}-14 clone, rather than the cumbersome "Korean variant of the Taiwan^{19F}-14 clone". This new suggested nomenclature allows the population structure of resistant pneumococci in New Zealand to be described more readily. This is because the Korea^{19F}-14 clone is not recognised as a PMEN clone, but rather a single locus variant of the Taiwan^{19F}-14 clone. Most of the variants of this clone in New Zealand are single locus variants of the Korea^{19F}-14 clone, which in turn makes them double locus variants of the Taiwan^{19F}-14 clone. It should be noted however, that apart from a single allele difference at the *aroE* loci, the Taiwan^{19F}-14 clone and Korea^{19F}-14 clone are indistinguishable. The Korea^{19F}-14 clone has the most dominant ST identified in the current study; use of this term better facilitates its description when discussing data obtained from MLST.

The New Zealand isolates contained five examples of the Taiwan^{19F}-14 clone (ST 236), as well as one single locus (*ddl*) variant (ST 237, isolate 98-537), two double locus (*gki* and *ddl*) variants (isolates 93-380 and 94-993) and one triple (*gki*, *xpt* and *ddl*) locus variant (isolate 93-612). These three isolates were recovered during 1993-94 and their PFGE type has not been observed since. Thirteen examples of the Korea^{19F}-14 clone (ST 271) were identified, as well as four unique single allele variants. Isolates 95-176, 98-370, 98-441 98-619 each differed from the Korea^{19F}-14 clone at the *xpt* (allele 20→1), *ddl* (allele 26→8), *ddl* (allele 26→85) and *spi* (allele 6→5), loci respectively (Table 4.6).

It should be noted that the Taiwan^{19F}-14 clone has a characteristic PFGE profile (Figure 4.4), which is identical to that described in the previous chapter as belonging to the major resistant pneumococcal clone in New Zealand. The major Taiwan^{19F}-14 strain and it variants appear to have become established in New Zealand in 1995 as no corresponding PFGE type has been noted before. The New Zealand Taiwan^{19F}-14 clonal group is resistant to erythromycin, co-trimoxazole, and tetracycline (with the exception of strains 93-380, 93-612 and 94-993 which are susceptible to co-trimoxazole) and uniformly susceptible to chloramphenicol and vancomycin (with the exception of 99-104, 00-499 and 00-716 which appear to have recently acquired resistance to chloramphenicol).

Levels of β-lactam resistance are quite variable among this group. In the case of the five members of the Taiwan^{19F}-14 clone, the penicillin and cefotaxime MICs are consistently 2 and 1 μg/ml (one instance of a cefotaxime MIC of 0.5 μg/ml) respectively. However, in the 14 Korea^{19F}-14 variants MICs vary from 1 to 8 μg/ml and 0.5 to 32 μg/ml for penicillin and cefotaxime respectively. This variability in the β-lactam MICs is not mirrored in the respective

pbp RFLP profiles. In all instances (with the exclusion of isolate 98-441) all the New Zealand members have identical *pbp* RFLP profiles. Isolate 98-441 however, has *pbp* RFLP profiles identical to those associated with the Spain^{23F}-1 and France^{9V}-3 clonal groups. Interestingly, this isolate is also unusual in that its cefotaxime MIC is quite low (0.5 μg/ml) as compared to its high penicillin MIC (4 μg/ml). As was seen with the France^{9V}-3 clonal group, a change in the *pbp* RFLP patterns was also associated with the acquisition of a new *ddl* allele (allele 26 → 85).

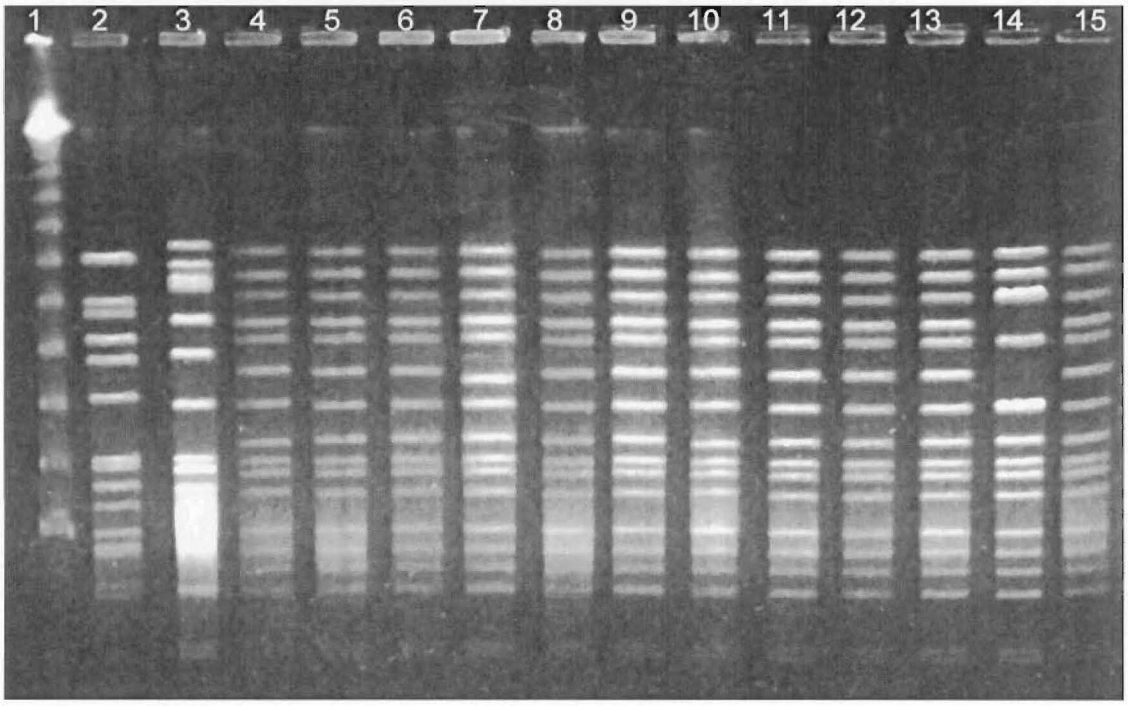

Figure 4.4 DNA macro-restriction profiles of selected serogroup 19 isolates analysed by MLST.

Lanes 1) Lambda ladder, 2) P95-066 (ST undesignated, serotype 19A), 3) A94-993 (ST undesignated, serotype 19F), 4) P97-076 (ST 236, serotype 19F), 5) P97-291 (ST 271, serotype 19F), 6) A98-370 (ST undesignated, serotype 19F), 7) A98-619 (ST undesignated, serotype 19F), 8) P97-626 (ST 271, serotype 19F), 9) A96-755 (ST 271, serotype 19F), 10) A97-066 (ST 236, serotype 19F), 11) A97-724 (ST 271, serotype 19F), 12) A98-441 (ST undesignated, serotype 19F), 13) A99-539 (ST 271, serotype 19F), 14) A99-1014 (ST 271, serotype 19F), 15) 99-909 (ST 271, serotype 14)

Strain[a]	Allelic profile							ST	MRP[b]	Sero	MIC[c]		RP[d]	*pbp*[e]	
	aro	*gdh*	*gki*	*rec*	*spi*	*xpt*	*ddl*				P	C		*2b*	*2x*
Taiwan^{19F}-14 clone															
97-006	15	16	19	15	6	20	26	236	1A	19F	2	1	E, S, T	A	A
97-503	15	16	19	15	6	20	26	236	1A	19F	2	1	E, S, T	A	A
97-066	15	16	19	15	6	20	26	236	1A	19F	2	1	E, S, T	A	A
97-076*	15	16	19	15	6	20	26	236	1A	19F	2	1	E[f]	A	A
98-120*	15	16	19	15	6	20	26	236	nd	19F	2	0.5	E[g]	A	A
98-537	15	16	19	15	6	20	**1**	237	nd	19F	0.5	16	E, S, T	A	A
Taiwan^{19F}-14 variants															
93-380	15	16	**2**	15	6	20	**new**	NA	nd	19F	2	1	E, S, T	G	G
94-993	15	16	**2**	15	6	20	**new**	NA	nd	19F	2	1	E, T	G	G
93-612	15	16	**2**	15	6	**5**	**new**	NA	nd	19F	4	1	E, S, T	nd	G
Korea^{19F}-14 clone															
95-061	4	16	19	15	6	20	26	271	1A	19F	2	1	E, S, T	A	A
95-235	4	16	19	15	6	20	26	271	1A	19F	2	1	E, S, T	nd	A
96-755	4	16	19	15	6	20	26	271	1A	19F	2	1	E, S, T	A	A
97-359*	4	16	19	15	6	20	26	271	1A	19F	4	1	E, S, T	A	A
97-635	4	16	19	15	6	20	26	271	1A	19F	4	2	E, S, T	A	A
97-291*	4	16	19	15	6	20	26	271	1A	19F	4	2	E, S[f]	A	A
97-626*	4	16	19	15	6	20	26	271	1A	19F	4	8	E, S[f]	A	A
97-724	4	16	19	15	6	20	26	271	1A	19F	8	32	E, S, T	A	A
99-539	4	16	19	15	6	20	26	271	1A	19F	4	8	E, S, T	A	A
99-909	4	16	19	15	6	20	26	271	1A	14	4	8	E, S, T	A	A
99-1014	4	16	19	15	6	20	26	271	1E	19F	4	4	E, S, T, C	A	A
00-499	4	16	19	15	6	20	26	271	1A	19F	4	8	E, S, T,	A	A
00-716	4	16	19	15	6	20	26	271	1A	19F	8	16	E, S, C	A	A
Korea^{19F}-14 variants															
95-176	4	16	19	15	6	**1**	26	NA	nd	19F	4	2	E, S, T	A	A
98-370	4	16	19	15	6	20	**8**	NA	1A	19F	4	1	E, S, T	A	A
98-441	4	16	19	15	6	20	**85**	NA	1A	19F	4	0.5	E, S, T	B	B
98-619	4	16	19	15	**5**	20	26	NA	1B	19F	4	1	E, S, T	A	A

Table 4.6 Allelic profiles, serotype and antibiotic resistance profiles of 26 clinical isolates related to the Taiwan^{19F}-14 clone.

Bold indicates variant alleles, (*a*) Isolates marked with asterisk from invasive disease, (*b*) MRP based on profiles described in chapter 3, (*c*) Penicillin, and cefotaxime (respectively) MICs in µg/ml, (*d*) Resistance profile, all isolates tested against erythromycin (E), co-trimoxazole (S), tetracycline (T) and chloramphenicol (C) unless stated otherwise, (*e*) RFLP pattern of PCR amplified *pbp* gene fragment, based on profiles described in chapter 3, (*f*) Resistance to tetracycline not determined, (*g*) Resistance to co-trimoxazole and tetracycline not determined.

4.2.2 Non PMEN groups identified by MLST

Fourteen isolates yielded allelic profiles that matched, or were single allele variants of those already in the MLST database (Table 4.7). These 14 isolates could be described as members of (or variants of) one of seven previously defined groups, serotype 9N, 14 (2), 19A (2), 19F or 23F respectively.

Strain[a]	Allelic profile							ST	Sero	MIC[b]		RP[c]	*pbp*[d]	
	aro	*gdh*	*gki*	*rec*	*spi*	*xpt*	*ldl*			P	C		*2b*	*2x*
93-075*	2	8	2	4	6	1	1	66	9N	0.016	0.016	--	D	E
95-298*	2	8	2	4	6	1	1	66	9N	0.03	0.016	--	G	E
94-055*	7	5	1	8	14	11	14	124	14	0.016	0.016	--	E	E
95-194*	7	5	1	8	14	11	14	124	14	0.016	0.016	--	E	E
94-001*	7	5	1	8	14	11	**5**	129	14	0.016	0.016	S	E	E
93-116*	7	5	1	**4**	14	11	14	NA[f]	14	0.016	0.016	E	E	E
95-255*	10	41	47	16	6	14	2	346	14	0.5	0.12	--	J	N
92-247*	8	13	14	4	17	4	14	199	19A	0.25	0.06	S	I	L
93-345*	8	13	14	4	17	4	14	199	19A	0.12	0.06	S	B	L
95-066*	8	13	14	4	**6**	4	14	NA[g]	19A	0.12	0.06	S	I	L
93-216*	7	**13**	1	1	14	28	31	NA[h]	19A	0.016	0.016	--	E	E
94-054*	7	6	1	2	6	15	14	146	19F	0.016	0.016	--	E	E
93-316*	1	8	4	1	1	4	6	36	23F	0.016	0.016	--	D	D
97-083*	1	8	4	1	1	4	6	36	23F	2	1	C[e]	nd	D

Table 4.7 Allelic profiles, serotype and antibiotic resistance profiles of strains for which there was an exact or near match in the MLST database, but were not related to PMEN designated clones.

Ordered by serotype. Bold indicates variant alleles, (*a*) Isolates marked with asterisk from invasive disease, (*b*) Penicillin, and cefotaxime (respectively) MICs in μg/ml, (*c*) Resistance profile, all isolates tested against erythromycin (E), co-trimoxazole (S), tetracycline (T) and chloramphenicol (C) unless stated otherwise (dashes indicate sensitivity to all four agents), (*d*) RFLP pattern of PCR amplified *pbp* gene fragment, based on profiles described in chapter 3, (*e*) Resistance to tetracycline not determined (*f*) single locus variant (*rec*) of ST 124, (*g*) single locus variant (*spi*) of ST 199, (*h*) single locus variant (*gdh*) of ST 186.

4.2.2.1 Serotype 9N: ST 66

Two serotype 9N isolates gave identical allelic profiles that corresponded with sequence type 66, which has been identified previously in Sweden and the UK from cases of pneumonia and bacteraemia. Both isolates were penicillin-sensitive, as were the other ST 66 isolates described.

Isolates recovered in 1993 and 1995, have similar although not identical PFGE patterns. A change in the RFLP profile of the *pbp2b* also occurred within the 1995 isolate, which may account for the doubling in penicillin MIC.

4.2.2.2 Two serotype 14 groups: ST 124 and 346

Two groups of sequence types both expressing serotype 14 were identified. The first group contained four isolates from invasive disease. Two of the isolates belonged to sequence type 124, and two were single allele variants; one had an altered *ddl* gene (ST 129) and the other an altered *recP* gene (undesignated ST). Sequence type 124 (and its derivatives) is the major serotype 14 clone associated with invasive disease. Isolates belonging to this type have been recovered throughout Europe, including Sweden, Denmark, Norway, Finland and the UK. Members of this clone have also been isolated outside Europe, in Australia and Canada, suggesting that it is globally distributed. This group is susceptible to all antibiotics tested, including β-lactams, and had conserved *pbp* RFLP profiles. All four strains had similar (not identical) PFGE patterns.

The second serotype 14 group identified by MLST contained one isolate and belonged to sequence type 346. Only one example of a sequence type 346 is listed in the MLST database, this belongs to a serotype 15C isolate from Norway. Furthermore, the isolate had a penicillin MIC of 0.25 μg/ml (intermediate) and was susceptible to erythromycin. In contrast, the ST 346 isolate (95-255) in the current study was serotype 14, suggesting transfer of capsular genes. Isolate 95-255 also had intermediate susceptibility to penicillin and was susceptible to erythromycin. The *pbp* RFLP and MRPs of this isolate were unique when compared to other New Zealand isolates.

4.2.2.3 Two serotype 19A groups: ST 199 and 186

Two groups both expressing serotype 19A were identified. The first group contained three isolates; two belonging to ST 199, and one single locus (*spi*) variant (undesignated sequence type). Sequence type 199 isolates have been recovered previously from invasive disease in the UK, although one ST 199 isolate expressing serotype 15B has been isolated from the Netherlands. All three New Zealand isolates belonging to this group have intermediate penicillin resistance and are susceptible to cefotaxime. These isolates were also resistant to co-trimoxazole, but susceptible to other non-β-lactam antibiotic groups tested. All three isolates had identical RFLP profiles for *pbp2x*, but some variability was observed in *pbp2b*, although this did not result in a change in MIC.

Isolate 93-216 is a single locus variant (*gdh*) of ST 186. A single example of this sequence type exists in the MLST database, an invasive serotype 19A isolate from Australia. Both New Zealand and Australian isolates had identical antibiotic resistance profiles; fully susceptible to penicillin and erythromycin. Isolate 93-216 displayed *pbp* RFLP profiles common with other penicillin-susceptible sequence types such as ST 124. This sequence type, having to date only been reported in Australia, could represent an invasive strain endemic to this region of the Pacific.

4.2.2.4 Serotype 19F: ST 146

One example of a ST 146 was identified. This isolate (94-054) expressed serotype 19F, yet the other two ST 146 isolates described in the MLST database are serotype 6B. Sequence type 146 has been identified previously in the UK and US. The UK isolate was susceptible to both penicillin and erythromycin, but the isolate from the US had intermediate resistance to penicillin. The New Zealand ST 146 isolate was sensitive to penicillin and all other antibiotics that it was tested against. It also shares the *pbp* RFLP patterns of the other pencillin-susceptible isolates.

4.2.2.5 Serotype 23F: ST 36

Two serotype 23F isolates representative of ST 36 were identified. ST 36 has been isolated previously in Canada and the UK. In each case these isolates were acquired from invasive disease. One of the New Zealand ST 36 isolates was susceptible to penicillin, as were all the ST 36 isolates in the MLST database. However the other of the two ST 36 isolates was resistant to penicillin (MIC 2 μg/ml) and had intermediate cefotaxime-resistance (MIC 1 μg/ml). Furthermore, this isolate also expressed resistance to chloramphenicol. The two New Zealand isolates were recovered in 1993 and 1997 respectively, suggesting that these resistance mechanisms were acquired by a ST 36 isolate relatively recently.

4.2.3 Pneumococcal isolates without significant MLST matches

Six undesignated allelic profiles, which do not have a strong resemblance to any ST, are shown in Table 4.8. This group of six isolates was recovered between 1993-95 and are predominantly serotype 19F.

Of particular interest are two isolates, 93-431 and 95-109, which have little resemblance to any entry in the MLST database, but appear to be members of the same clonal group. These two isolates are both serotype 19F, have similar MICs, identical resistance profiles, common MRPs

and *pbp* RFLP patterns. This clonal group was isolated prior to 1995, but subsequent to that date it was observed less frequently and the Taiwan^{19F}-14 clone became more prominent. Both these isolates are phenotypically identical to the Taiwan^{19F}-14 clone, but are quite distinct genotypically.

None of the isolates shown in Table 4.8 currently has a matching ST in the MLST database. These sequences have been submitted for inclusion in the database, but due to the stringent standards maintained by the curator of the database, several loci have had to be re-sequenced. Owing to the high cost involved with automated DNA sequencing, this has not been performed to date. Furthermore, for the completion of this study it is not necessary for the isolates to be included in the MLST database. However for the benefit of pneumococcal researchers worldwide it would be beneficial to have MLST data from New Zealand included. It is anticipated that the necessary repeat sequencing will be performed, and the results made available to the research community through the MLST database.

Strain[a]	Allelic profile							ST	Sero	MIC[b]		RP[c]	*pbp*[d]	
	aro	*gdh*	*gki*	*rec*	*spi*	*xpt*	*ddl*			P	C		*2b*	*2x*
94-271*	18	3	4	18	15	1	14	NA	19[e]	0.016	0.016	--	nd	E
93-431	7	6	9	2	1	4	67	NA	19F	2	1	E, S, T	F	F
95-109	7	6	9	2	1	4	67	NA	19F	4	1	E, S, T	F	F
93-060*	7	5	19	16	14	14	28	NA	19F	1.0	0.12	--	nd	M
93-308*	10	13	34	16	15	1	4	NA	19F	0.016	0.016	T	E	E
93-295*	2	2	9	2	6	4	14	NA	23F	0.12	0.12	--	K	K

Table 4.8 Allelic profiles, serotype and antibiotic resistance profiles of isolates for which there was no near match in the MLST database. These isolates represent newly identified sequence types.

Ordered by serotype, (*a*) Isolates marked with asterisk from invasive disease, (*b*) Penicillin, and cefotaxime (respectively) MICs in μg/ml, (*c*) Resistance profile, all isolates tested against erythromycin (E), co-trimoxazole (S), tetracycline (T) and chloramphenicol (C) unless stated otherwise (dashes indicate sensitivity to all four agents), (*d*) RFLP pattern of PCR amplified *pbp* gene fragment, based on profiles described in chapter 3, (*e*) Not serotyped, only determined to group.

4.3 Discussion

In the short time since it has been described MLST has proved itself to be a useful tool in the global tracking and epidemiology of various bacterial pathogens. MLST was initially validated with the pneumococcus using 20 well-defined isolates including one *Streptococcus mitis* strain (Enright and Spratt, 1998). Four isolates of the Spain^{23F}-1 clone, two of which were capsular variants (19A and 19F respectively), were shown to be identical by MLST, as were three

reference isolates of the Spain^{6B}-2 clone. The remaining twelve isolates have been previously characterised by MLEE and PFGE analysis (Hall et al., 1996). The groupings obtained by MLST proved to be highly congruent with the results obtained by MLEE and PFGE, with the exception of two isolates. A serotype 9N and 9V isolate were not related by MLST (different at 6/7 loci), yet they differed at only 2/10 loci by MLEE. The two isolates were not however, considered related by PFGE (Hall et al., 1996).

MLST is well-suited to tracking the occurrence of clonal strains worldwide. This is of particular importance in the identification of emerging antibiotic-resistant, or hyper-virulent clones. Application of MLST to 274 pneumococci isolated globally from invasive disease with no pre-selection for serotype or antibiotic resistance, identified 143 isolates, 34 of which were represented more than once (Enright and Spratt, 1998). Of these, twelve groups, which were represented with five or more isolates, were defined as virulent clones. Of the twelve virulent clones, seven were found in at least two continents, and four clones were found on three continents (Enright and Spratt, 1998).

Seventy-four multi-drug resistant pneumococcal isolates from Taiwan were analysed by MLST (Shi et al., 1998). Of the 74 isolates, 86% could be grouped into one of three penicillin-resistant clones. Of these three groups, 29 isolates belonged to the Taiwan^{19F}-14 clone, 21 isolates belonged to the Spain^{23F}-1 clone, and 15 isolates belonged to the Taiwan^{23F}-15 clone. Two of these clones were unique to Taiwan, but nevertheless, the Spain^{23F}-1 clone was still fairly dominant. This demonstrates yet again the capacity of the Spain^{23F}-1 clone to travel between countries, and the utility with which MLST was used to differentiate it from endogenous resistant clones. The utility of MLST to detect pneumococcal clones was further demonstrated by Zhou and colleagues (Zhou et al., 2000). These investigators showed that predominant clones (Spain^{23F}-1, Spain^{6B}-2, France^{9V}-3 and Spain^{14}-5) could be identified throughout the world. Although in some instances phenotypically different (e.g., the Spain^{23F}-1 clone may express serotype 19F or 19A; the France^{9V}-3 clone may be penicillin-susceptible or resistant) the allelic profiles are well conserved among the clonal groups.

The use of MLST in conjunction with other molecular methods of bacterial strain identification allows the molecular epidemiological data presented in the previous chapter to interpreted with more clarity. The designation of pneumococcal isolates into groups based on their MRPs is useful for local molecular epidemiological analysis. Indeed it identified groups of isolates that appeared clonal in nature, and allowed temporal changes in the population structure to be

monitored. Major clonal groups identified by MRP analysis could be tentatively identified as belonging to certain worldwide clones, but such designations are haphazard, being based largely on phenotypic characteristics. Using this data in conjunction with MLST allows information generated through conventional molecular epidemiological methods to be interpreted globally.

4.3.1 Origin of penicillin-resistant pneumococci in New Zealand

Fifty-eight (78%) of the 74 New Zealand pneumococcal isolates in this study were able to be assigned to one of 15 STs. Furthermore, ten of the remaining 16 isolates were either single (7), double (2) or triple (1) allele variants of previously described STs. The remaining six isolates differed from all isolates previously described by MLST in >3 alleles. Of the 68 isolates that did have close or exact matches in the MLST database, 48 (71%) could be designated into one of three clonal groups.

Not surprisingly, two of the major drug resistant pneumococcal clones identified in New Zealand were determined to be members of the Spain^{23F}-1 clone and the France^{9V}-3 clone. Theses two clones accounted for 19% and 15% of the isolates characterised by MLST, respectively. Previous MLST studies have shown the widespread global distribution of these clones (Shi et al., 1998, Zhou et al., 2000). Due to worldwide prevalence of these clones, it is not possible to determine an origin from where these isolates may have been imported.

The same cannot be said for the 23 isolates belonging to the Korea^{19F}-14 clonal group. Having been described only recently, members of this group have only started to disseminate globally over the last few years. The first isolate of this clone identified in New Zealand was recovered in 1995, yet the members of the clone had been recovered in Asia as early as 1993. This suggests this clone may have originated in Asia, and subsequently been imported into New Zealand.

Until relatively recently, the issue of antibiotic resistance has not received a great deal of attention in Asia. The formation of Asian Network for Surveillance of Resistant Pathogens (ANSORP) in 1996 has revealed that levels of antibiotic-resistance in Asian countries are among the highest in the world (Song et al., 1999). Consequently, the emergence of highly resistant strains of *S. pneumoniae* in Asia is not unexpected, given recent reports of resistance in this part of the world. A multi-centre study conducted in 1996-1997 found the prevalence of penicillin resistance in the pneumococcus to be as high as 79.9% in Korea (Song et al., 1999). Other countries with notably high resistance include Japan (65.3%), Vietnam (60.8%) and

Thailand (57.9%). Taiwan was reported to have a prevalence rate of 38.7%. These resistance rates are extremely high and are quite in excess of rates being reported in other parts of the world, including the well-identified centres of pneumococcal resistance such as Hungary, Spain and France (see Figure 1.1 for comparison). The main serotypes of resistant pneumococci isolated throughout Asia are 23F and 19F (Song et al., 1999).

The level of penicillin non-susceptible *S. pneumoniae* carriage seen in a Taiwanese paediatric population has been shown to be 71% (Chiou et al., 1998). The same study reports comparatively high rates of resistance to other antibiotics as well, most notably the macrolide antibiotic clarithromycin, to which 95% of the isolates showed resistance. A similar report observed resistance rates of 82% and 90% to erythromycin and clarithromycin respectively (Hsueh et al., 1999). This high level of macrolide-resistance is attributed to the availability of this class of antibiotic that can readily be acquired without prescription (Chiou et al., 1998, Hsueh et al., 1999).

The Taiwan^{19F}-14 clone is the major multiply antibiotic-resistant strain of *S. pneumoniae* in New Zealand. The earliest confirmed identification of this clone in New Zealand is 1995, which coincides with a period of increased prevalence of penicillin-resistant *S. pneumoniae*. It seems likely, therefore, that the increase is due to the importation and subsequent dissemination of the Taiwan^{19F}-14 clone. This follows what has been observed elsewhere – the most notable example being Iceland (Soares et al., 1993). Iceland is similar to New Zealand in that both countries are geographically isolated and resistant strains had to literally be imported. Yet once established the resistant strains are quite effective at displacing the natural population and spreading clonally.

When the serotype 6B multi-resistant clone spread from Spain into Iceland, several factors were suggested that might have facilitated its spread. The large proportion (57%) of the country's population living in the city of Reykjavik; the high proportion (77%) of children in Reykjavik attending day care centres; and the popularity of Spain as a holiday destination may have contributed to the importation and spread of the Spain^{6B}-2 clone in Iceland (Soares et al., 1993).

Some parallels can be drawn between the situations in Iceland and New Zealand. New Zealand has been actively promoting its tourist industry during the last decade, which has led to an increase in international visitors to New Zealand. Furthermore, New Zealand has one of the highest per capita international travel rates in the world, with Oceania and Asia representing the

two most popular short-term travel destinations (Crump et al., 2001). New Zealand also accepts more than 50 000 immigrants annually, predominantly from the same regions (Crump et al., 2001). Between 1991 and 1996 New Zealand's overseas-born population increased by almost 78,000 people, with 72% born in Asia (Statistics New Zealand, 2000). Interestingly, when the data from the 1991 and 1996 New Zealand census are compared, the two biggest increases were in people born in Korea and Taiwan (Table 4.9).

Country of Birth	1991 Census		1996 Census		% Intercensal change
	Number	*%*	*Number*	*%*	
New Zealand	2 812 035	84.2	2 848 209	82.5	1.3
Australia	48 636	1.5	54 575	1.6	12.2
UK/Ireland	239 157	7.2	230 052	6.7	-3.8
South Korea	801	<0.1	12 183	0.4	1 421.0
Taiwan	3 411	0.1	10 932	0.3	220.5
Malaysia	8 820	0.3	11 889	0.3	34.8
China	9 225	0.3	19 518	0.6	111.6
Hong Kong	4 929	0.1	11 760	0.3	138.6
India	9 456	0.3	12 807	0.4	35.4
Other Asia	25 473	0.8	38 706	1.1	51.9
Total	3 373 923	100	3 618 306	100	7.2

Table 4.9 Country of birth of NZ population (adapted from (Statistics New Zealand, 2000)).

This increase in population of persons born overseas coincides with the time the Taiwan^{19F}-14 clone is thought to have arrived in New Zealand. Although the absolute numbers of people born either Korea or Taiwan is not as high as those of Australia and United Kingdom (two highest) the percentage increase in a short period of time is quite dramatic. This rapid increase could have allowed a theoretical threshold at which an exogenous microorganism could become integrated into the community, to be exceeded.

The Taiwan^{19F}-14 clone is apparently a fairly recent clone to emerge; the first formal identification made when multiply antibiotic-resistant *S. pneumoniae* from Taiwanese hospitals were examined by MLST (Shi et al., 1998). This major 19F clone was present in Taiwan as early as 1993. In 1995, two serotype 19F isolates were recovered from blood cultures in a London hospital that had the same allelic profile. This is the same year that the Taiwan^{19F}-14 clone was observed in New Zealand. Therefore it seems likely the Taiwan^{19F}-14 strain did in

fact originate in Taiwan, or elsewhere in Asia, and began to disseminate globally as early as 1995.

4.3.2 Penicillin resistance and sequence type

In several strains analysed by MLST, most notably the serotype 9V strains, a change from penicillin-sensitivity to resistance was accompanied by a change in ST from 162 (sensitive) to 156 (resistant). This change in ST was associated with a single allele change of the *ddl* gene, encoding D-alanyl-D-alanine ligase. This is a cytoplasmic enzyme involved in the biosynthesis of a peptidoglycan precursor. The *ddl* gene itself is located 783 bp downstream of another gene whose product is involved in peptidoglycan biosynthesis, *pbp2b*. The possibility that the *ddl* gene "hitchhikes" with the *pbp2b* gene under β-lactam selection was explored by Enright and Spratt, 1999 (Enright and Spratt, 1999a). They found 52 different *ddl* alleles in the entire pneumococcal MLST database of 566 submissions (as of 1999). Analysis of the sequence showed that these alleles could be divided into two groups. The first group was uniform and was found in both sensitive and resistant isolates. The second group showed sequence diversity and were only present in penicillin-resistant isolates. Hitchhiking of the *ddl* gene was shown to occur in three New Zealand isolates (94-015, 94-115 and 95-213). This was further confirmed by the *pbp2b* RFLP analysis of these strains – ST 162 has a RFLP pattern (type D) quite different from the ST 156 (type B).

4.3.3 Serotype switching in the pneumococcus

Changes in capsular types are a phenomenon MLST is well-suited to detecting. Switching of serotype in the pneumococcus is a well-documented occurrence. The Spain^{23F}-1 clone has, for example, been found to express serotypes 3, 9N, 14 and 19F (Coffey et al., 1991, Nesin et al., 1998). Serotype switching seems to be due to movement of "cassettes" containing capsular genes between organisms. The required genes to synthesize a given serotype may be uptaken (presumably via natural transformation) and recombine in the region of the original capsular genes. This may result in the loss of the original capsular genes as demonstrated on a group of isolates belonging to the Spain^{23F}-1 clone, but expressing serotypes 19, 14, or 3. These isolates were tested using DNA probes for each of the 18 open reading frames (ORFs) of the 23F capsular locus. In no case were there any 23F-specific genes retained, with the possible exception of genes already known to be common to the capsular loci involved (Ramirez and Tomasz, 1999).

Five isolates among those analysed by MLST showed evidence of having switched their capsular type. One Spain^{23F}-1 clone (ST 81) was found to express serotype 14 (isolate 97-036) and two were of serotype 19F (isolates 98-283 and 00-058). In each case these three isolates resembled the Spain^{23F}-1, but for the change in serotype. As stated previously, members of the Spain^{23F}-1 clone have been found to express multiple capsular types, including 14 and 19F (Coffey et al., 1991, Nesin et al., 1998), so this observation is not necessarily surprising. Two members (isolates 98-286 and 98-555) of the France^{9V}-3 clone (ST 156) were found to belong to serotype 14. This transfer has also been documented previously (Coffey et al., 1999). One member (99-909) of the Korea^{19F}-14 (ST 271) was found to express serotype 14, rather than 19F. This is of particular concern, as this serotype is one more frequently associated with invasive disease (Shapiro and Austrian, 1994). The predominant Korea^{19F}-14 clone in New Zealand is highly antibiotic-resistant, consequently increased invasive disease due to this clone could pose an important public health problem. A final example of serotype switching was noted in isolate 94-054, belonging to ST 146. This sequence type is associated with an invasive, serotype 6B clone, recovered from patients in the UK and US. The implications of this particular transfer are not immediately obvious. However, given the rapid spread of the Korea^{19F}-14 clone in New Zealand, perhaps this capsular type lends itself more readily to colonisation of new hosts.

It should be noted that in each of the seven isolates in which serotype switching was observed, all other genotypic and phenotypic traits (e.g., antimicrobial resistance, PFGE profiles etc) were consistent with the parental strain. This is of particular concern if capsular types associated with invasive disease are transferred to highly antibiotic-resistant isolates. Such transfer has been observed between New Zealand isolates, and could potentially mark the beginning of very difficult to treat, serious pneumococcal infections.

Chapter 5

Pneumococcal β-Lactam Resistance

5.1 Introduction

A notable feature of the penicillin-resistant pneumococci isolated in New Zealand is their association with high-level resistance to cephalosporins. Typically, a penicillin-resistant isolate will have a cefotaxime MIC lower than its respective penicillin MIC. However, in New Zealand, a large proportion of the penicillin-resistant isolates that have been recovered have cefotaxime MICs which exceed their penicillin MICs (Brett, 2001). Highly cefotaxime-resistant (for the purposes of this study a cefotaxime MIC ≥ 4 μg/ml will be referred to as highly resistant) isolates were first detected in New Zealand in 1997 (Brett, 1997). In the current study, 30 of the 200 Christchurch isolates exhibited high-level resistance to cefotaxime. When the MRPs of the 30 highly cefotaxime-resistant isolates were examined, it was noted that resistance was associated with the MRP of the Korea^{19F}-14 clone (Figure 5.1).

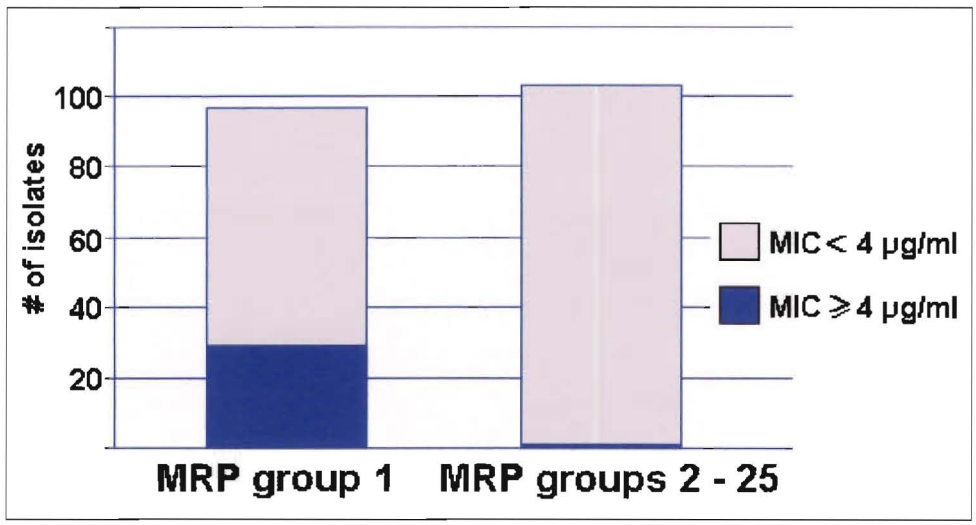

Figure 5.1 Proportion of Christchurch isolates exhibiting high-level cefotaxime-resistance (MIC ≥ 4.0 μg/ml) associated with MRP group 1 and non group 1.

The major 19F clone in New Zealand has been shown to be genetically homogenous (Chapter 3). Characterisation of isolates by MLST (Chapter 4) enabled variants from Korea and Taiwan to be distinguished from one another. The largest degree of variability observed in this group,

however, is in a specific phenotype; resistance to β-lactams. A wide range of MICs of penicillin and cefotaxime were observed in New Zealand members of this clone. Penicillin MICs ranged between 0.5 and 8.0 μg/ml and cefotaxime-resistance was more variable still, with MICs between 0.5 and 16 μg/ml observed. Furthermore, the distribution of MICs was unexpected. While the range of penicillin MICs formed a normal distribution with a median MIC of 2 μg/ml, the range of cefotaxime MICs formed a bimodal distribution, with one resistance peak at 1-2 μg/ml, and a second peak at 8 μg/ml (Figure 5.2). This chapter describes the analysis of the β-lactam resistance mechanism in the New Zealand variant of the Taiwan^{19F}-14 clone in more detail.

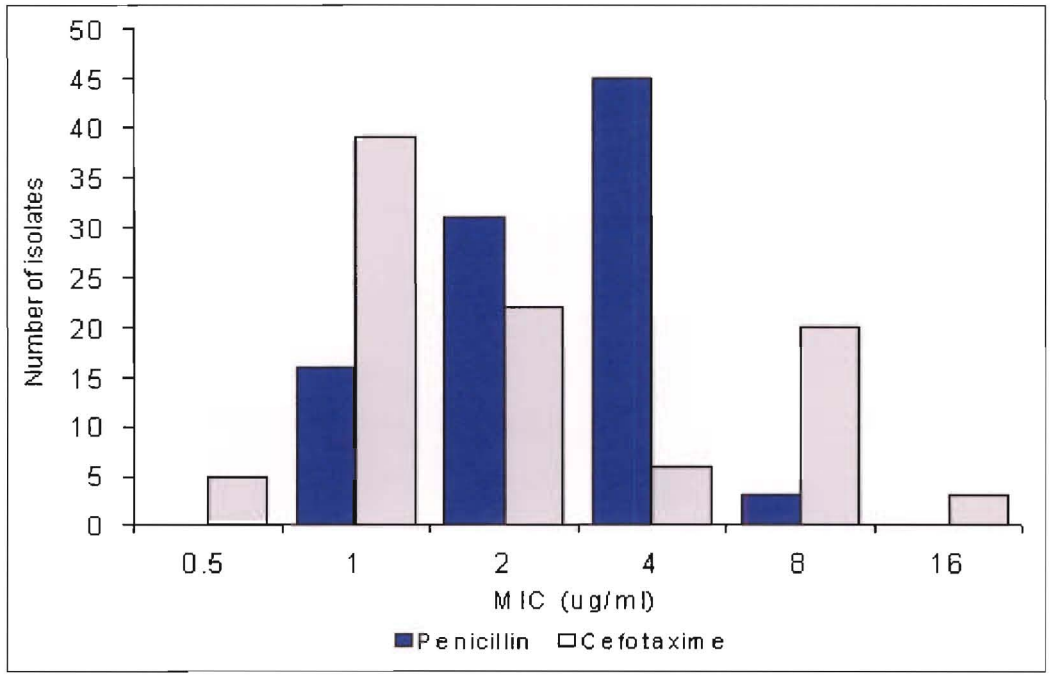

Figure 5.2 Frequency of the penicillin and cefotaxime MICs among the 97 Christchurch isolates of the New Zealand 19F clone.

5.1.1 Mechanism of β-lactam resistance

As discussed in Chapter 1, the development of β-lactam resistance in the pneumococcus is attributed mainly to alteration of the target enzymes, the penicillin binding proteins (PBPs) (Coffey et al., 1995b). Resistant PBPs have alterations that reduce their affinity toward penicillin, as shown initially by Tomasz and co-workers (Hakenbeck et al., 1980, Zighelboim and Tomasz, 1980). These investigators incubated pneumococcal protein extracts with radioactively labelled penicillin, then performed SDS-PAGE and autoradiography. This demonstrated that the PBPs of resistant pneumococci bound less [^{3}H]-penicillin than PBPs of susceptible pneumococci (Hakenbeck et al., 1980). It was noted that there were at least five such

proteins that bound penicillin in the sensitive pneumococcus, and in some cases a sixth (Hakenbeck et al., 1980). Subsequently the PBPs were named PBP1a, 1b, 2a, 2b, 2x and 3, in order of descending molecular weight. By using a range of concentrations of radiolabelled penicillin, it was possible to determine which of the PBPs was most susceptible, and what concentration of the antibiotic was required to fully saturate any given PBP target (Hakenbeck et al., 1980). It was also soon established that different PBPs had differing affinities for antibiotics (Williamson et al., 1980). Furthermore, the low affinity PBPs could be introduced into susceptible pneumococci by successive transformation experiments with DNA of a resistant isolate (Zighelboim and Tomasz, 1980).

The cloning of *pbp* genes allows the contribution of individual PBPs in penicillin resistance to be examined. A cloned *pbp* gene (or gene fragments amplified by PCR) from a resistant isolate was introduced into a sensitive strain (typically strain R6) and the resistance phenotype (MIC) imparted by the introduced genes was measured. Using this methodology it was shown that certain *pbp* genes were more important in imparting resistance than others. Alterations in the gene encoding PBP2B were shown to mediate low-level resistance to penicillin (Grebe and Hakenbeck, 1996, Krauss et al., 1996), while changes to PBP2x mediated low-level resistance to cephalosporins (Munoz et al., 1992, Grebe and Hakenbeck, 1996, Krauss et al., 1996). High-level resistance to either class of drug was imparted by subsequent alterations of PBP1A (Reichmann et al., 1996).

The application of DNA sequencing technology showed that the alterations in the PBPs could be directly attributed to changes in the nucleotide sequence of the genes encoding them (Dowson et al., 1989b). Examination of the nucleic acid sequence of *pbp* genes from resistant isolates revealed replacement of segments of these genes with corresponding regions from those of closely related bacteria (Dowson et al., 1994). These resistant genes are referred to as "mosaic" genes, and their formation believed to be facilitated by the natural competence of *S. pneumoniae*. The mosaic *pbp1a*, *pbp2b* and *pbp2x* genes of penicillin-resistant pneumococci contain regions from the corresponding genes of at least three different streptococcal species (Dowson et al., 1989a, Laible et al., 1991, Martin et al., 1992b).

5.1.2 Methods of investigating β-lactam resistance

The "classical" methods used to determine the mechanism of resistance, as described in the previous section, remain the most effective for investigation of β-lactam resistance.

5.1.2.1 Transformation

S. pneumoniae is a naturally competent bacterium. Non-encapsulated strains will readily take up DNA from their environment, and depending on its similarity to host pneumococcal DNA, it may be recombined and expressed. Transformation of susceptible strains with mosaic *pbp* genes has previously been shown to provide a system by which the role that individual *pbp* genes play in β-lactam resistance may be elucidated (Munoz et al., 1992, Coffey et al., 1995a, Grebe and Hakenbeck, 1996). Recipient strains are unencapsulated to help facilitate the transformation process. Encapsulated strains have been transformed by addition of exogenous CSP (Yother et al., 1986).

5.1.2.2 PBP β-lactam affinity

Affinity of individual PBPs toward penicillin can be determined by preparation of crude pneumococcal cell wall extracts and incubation in the presence of labelled penicillin. Penicillin forms a stable covalent linkage to the active site serine residue, allowing PBPs to be separated and identified by conventional SDS-PAGE. Previous studies have employed ^{3}H-, ^{14}C-, or ^{125}I-labelled penicillin to detect PBPs. To avoid the need to use radioactivity, and the problems associated with working with radioactive materials, a recently described, commercially available fluorescent penicillin; Bocillin FL (Zhao et al., 1999) was used. Bocillin FL is a fluorescent derivative of penicillin V, which fluoresces at 511 nm upon excitation at 504 nm. The chemical structure of this compound is given in Figure 5.3.

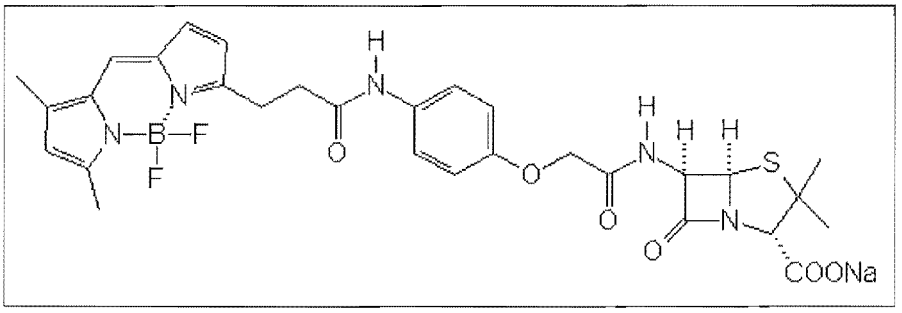

Figure 5.3 Chemical structure of BOCILLIN FL

The utility of this compound to detect penicillin-binding proteins was assessed by incubating pneumococcal protein extracts with increasing concentrations of the labelled penicillin to determine the MIC of each individual PBP (estimated as the minimum concentration of drug needed to bind and label each PBP). Based on the effectiveness of the fluorescent penicillin, competition experiments were also to be performed. These involve first incubating protein extracts with defined concentrations of β-lactam drugs (e.g., cefotaxime) and then labelling with

an excess of Bocillin FL. This enabled the primary targets of various β-lactam drugs to be elucidated.

5.1.2.3 Analysis of pbp *gene DNA sequence*

The formation of mosaic *pbp* genes necessarily involves alteration of their nucleotide sequences. This mosaicism can be exploited to document the differences in these genes. This can be done in one of two ways; directly, by determining the nucleotide sequence to the genes of interest; or, indirectly by exploiting the altered distribution of restriction endonuclease cleavage sites in these mosaic genes encoding PBP. The latter method (*pbp* RFLP) was used previously in chapter 2 and 3. The current chapter builds on the *pbp* RFLP data, and includes nucleotide sequence data to support observations.

5.2 Results

5.2.1 β-lactam agar dilutions

To further examine the variability of β-lactam resistance in the Taiwan^{19F}-14 clone, the MICs of seven β-lactam antibiotics was determined for 43 clinical isolates. Isolates were taken from throughout the country, including several of those included in chapter 3. All isolates included belonged to MRP group 1. The seven different β-lactams and their molecular structures are shown in Figure 5.4.

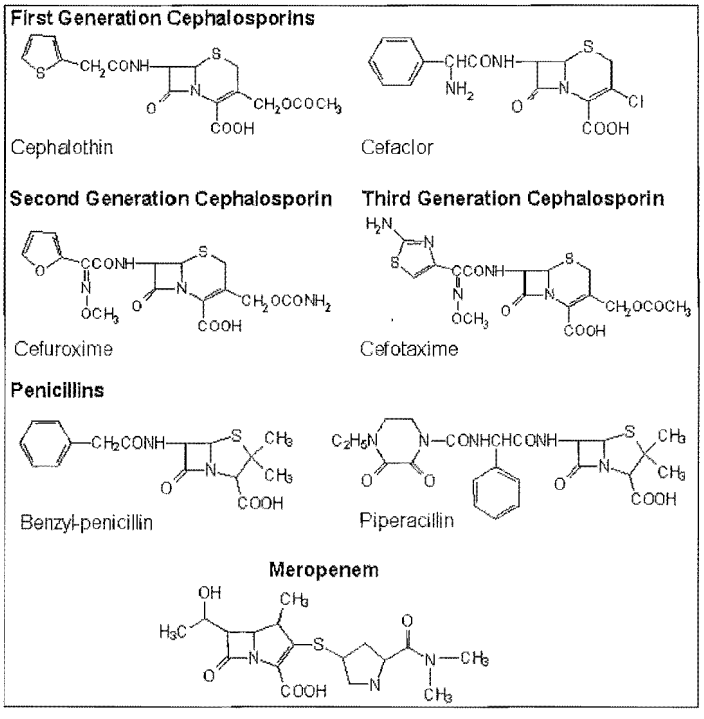

Figure 5.4 Chemical structure of the seven β-lactam antibiotics used in agar dilution determination of MIC.

Raw data for the β-lactam MIC determination are given in Appendix IV, a summary is shown in Table 5.1. All of the isolates tested were of either intermediate (7%) or fully (93%) penicillin resistance. The MIC range of piperacillin was to be narrower than that of penicillin, although for both compounds the MIC_{50} was 4 μg/ml. The MICs of the cephalosporin antibiotics ranged considerably between the 1st, 2nd and 3rd generation agents. All 43 isolates were resistant to cefaclor (MIC ≥ 64 μg/ml). The MICs of cephalothin followed a normal distribution centered around a MIC_{50} of 16 μg/ml. Cefuroxime and cefotaxime both had bimodal distributions. In each case a peak of 16 isolates had MICs of 4-8 μg/ml and 1 μg/ml respectively for cefuroxime and cefotaxime. In each case a second peak was observed at 32 μg/ml and 8 μg/ml respectively. All isolates had a reduced susceptible to meropenem compared to control strain ATCC 49619. Meropenem MICs ranged between 0.5 and 1.0 μg/ml, which exceed than the suggested meropenem breakpoints (Pikis et al., 1997, Fitoussi et al., 1998).

It is interesting to note differences between the MICs determined by agar dilution and Etest. Although results were comparable (both give MIC_{50} of 4 μg/ml and 8 μg/ml for penicillin and cefotaxime, respectively), some variation was observed at the resistant end of the spectrum. Etests tend to approximate higher MICs, the MIC_{90} for penicillin is 4 μg/ml and 8 μg/ml when measured by agar dilution and Etest respectively. A similar result is observed for cefotaxime, the MIC_{90}s are 8 μg/ml and 16 μg/ml when measured by agar dilution and Etest respectively. The MIC data was used to select isolates for further analysis.

Method	Antibiotic	MIC (ug/ml)										
		≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	≥64
Agar Dilution	Penicillin	ND	--	--	1 *	2	13	27	--	ND	ND	ND
	Piperacillin	ND	--	--	-- *	--	4	29	10	--	--	ND
	Cefaclor	ND	ND	--	--	--	-- *	--	--	--	--	43
	Cephalothin	ND	ND	--	--	-- *	--	1	6	19	17	--
	Cefuroxime	ND	ND	--	-- *	--	--	9	7	1	25	1
	Cefotaxime	ND	-- *	--	--	16	--	1	25	1	--	--
	Meropenem	-- *	--	--	18	25	--	--	--	ND	ND	ND
Etest**	Penicillin	--	--	--	1 *	--	10	25	5	--	--	--
	Cefotaxime	--	-- *	--	4	9	3	3	10	11	1	--

Table 5.1 Distribution of MICs of seven β-lactam antibiotics of 43 *S. pneumoniae* isolates belonging to MRP group 1.
ND not determined, * ATCC 49619 MIC to respective agent **two strains did not have Etests performed

5.2.2 *pbp* RFLP analysis

It was observed in Chapter two that the Taiwan^{19F}-14 clone was not always associated with the same *pbp2x* RFLP type. Although the profiles were typically RFLP type A, occasionally an isolate would produce type B profiles. To investigate this heterogeneity further, 55 isolates of this clone had *pbp2x* RFLP analysis performed using an additional two enzymes. For each of the three enzymes (*Dde*I, *Hinf*I and *Rsa*I) two different restriction profiles were generated (Figure 5.5).

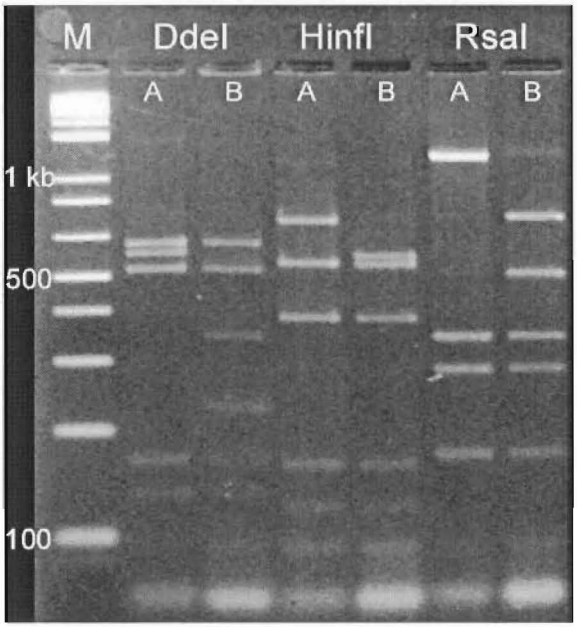

Figure 5.5 Representative *pbp2x* RFLP profiles of MRP group 1 isolates generated with three restriction endonucleases.

The distribution of different RFLP patterns is presented in Table 5.2. The most commonly observed combination of profiles (A, A, A) occurred in 83.6% of the MRP group 1 isolates. However, the MIC range for both penicillin and cefotaxime was variable in isolates exhibiting these profiles. For the remaining 16.4% of the isolates, various combinations of profiles were observed (Table 5.2). Although no obvious correlation between RFLP profiles and either penicillin of cefotaxime MIC was noted, this did serve to show the variability that exists within this gene. This suggested that by using a method that would provide greater resolution (e.g., determination of DNA sequence), such a correlation might be observed. To test this the genes *pbp1a*, *2b* and *2x* had their partial nucleotide sequence determined.

pbp2x RFLP profile			Frequency (%)	MIC (μg/ml)	
*Dde*I	*Hinf*I	*Rsa*I		Pen	Cef
A	A	A	46 (83.6)	2 - 8	0.5 – 16
A	A	B	1 (1.8)	4	2
A	B	A	1 (1.8)	2	2
A	B	B	1 (1.8)	4	8
B	A	A	1 (1.8)	4	2
B	B	A	3 (5.6)	1 - 2	1 - 2
B	B	B	2 (3.6)	4	0.5 – 1

Table 5.2 Distribution of *pbp2x* RFLP profiles generated by three different restriction endonucleases, and their associated β-lactam MICs.

5.2.3 Nucleotide sequence of *pbp* genes

The nucleotide sequence of three *pbp* genes, *1a, 2b,* and *2x,* was determined in four, six and eleven isolates, respectively. All DNA sequencing was performed on New Zealand isolates of the Taiwan^{19F}-14 clone, as shown in Table 5.3. See Table 2.4 for descriptions of the primers used for DNA sequencing. Isolates were chosen to give the greatest range of β-lactam resistance as described in section 5.2.1.

Isolate	*pbp* genes sequenced	Penicillin MIC (μg/ml)	Cefotaxime MIC (μg/ml)
98-537	*2b, 2x*	0.5	16
98-120	*2x*	2	0.5
98-491 (Sp202)	*1a, 2b, 2x*	2	0.5
98-441 (Sp194)	*2b, 2x*	4	0.5
97-816 (Sp038)	*2x*	4	1
98-618 (Sp272)	*1a, 2b, 2x*	4	1
95-176 (Sp042)	*2x*	4	2
98-560 (Sp262)	*2x*	4	8
99-539	*1a, 2b, 2x*	4	8
99-205 (Sp419)	*2x*	4	16
99-206 (Sp421)	*1a, 2b, 2x*	8	16

Table 5.3 Isolates of the Taiwan^{19F}-14 that had the nucleotide sequence of their *pbp* genes determined, and their respective penicillin and cefotaxime MICs.

5.2.3.1 Partial pbp1a *gene sequence*

The nucleotide sequence of the *pbp1a* gene from five pneumococcal isolates was determined. One of the five isolates was sensitive to penicillin (96-079), and the remaining four were resistant. DNA sequence was determined for an 1105 bp region of the four resistant isolates, and an 1012 bp region for isolate 96-079. These regions covered nucleotides 1880-2985 and 1959-2971 of the transpeptidase region respectively. The predicted amino acid sequences derived

from these regions of DNA are shown in Figure 5.6. Within the 1.1 kb region of the four resistant isolates, 200 (18.1%) nucleotide substitutions, resulting in 42 amino acid changes, were noted when compared to sensitive strain R6. Nucleotide substitutions were noted in all three of the conserved motifs (shaded), but only one resulted in an amino acid change; Thr-371→Ser By comparison, there were only eleven nucleotide substitutions noted in the *pbp1a* gene of isolate 96-079, resulting in seven amino acid changes.

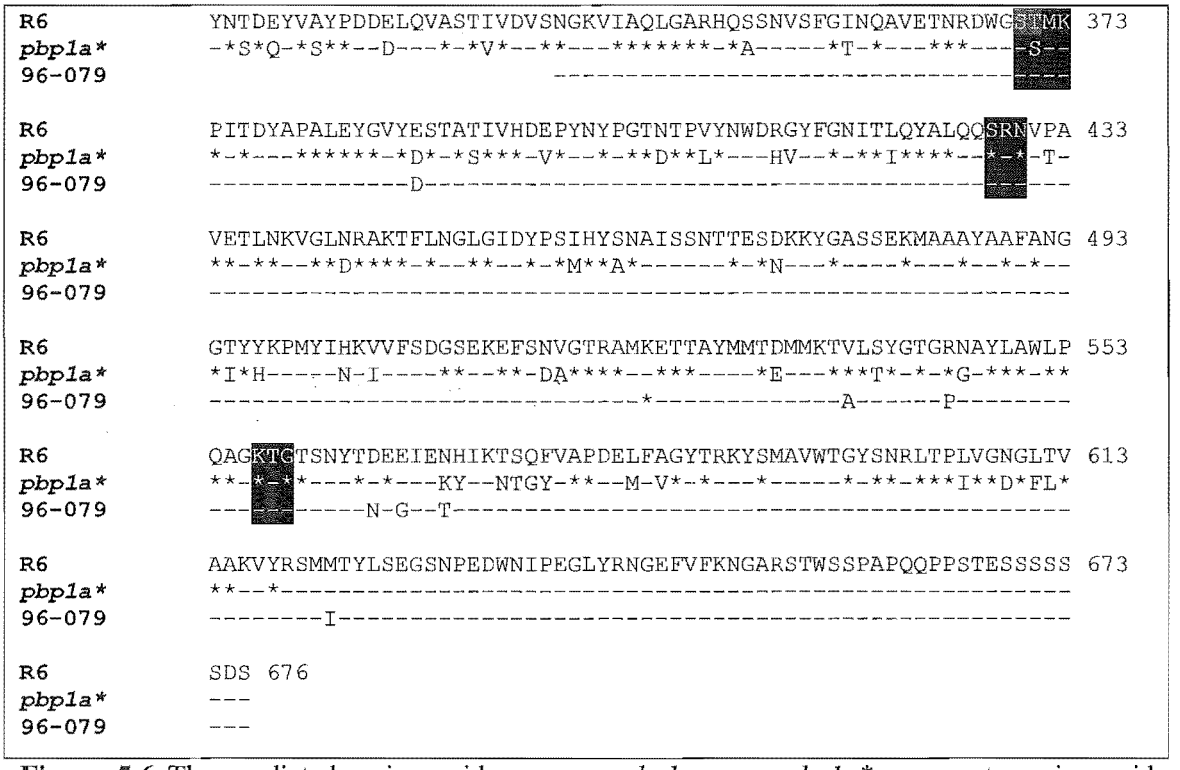

Figure 5.6 The predicted amino acid sequence *pbp1a* gene. *pbp1a** represents amino acid sequence of all four resistant isolates.

Shaded regions represent conserved motifs, dashes (-) indicate the amino acid sequence is identical to R6, asterisks (*) indicate a silent substitution in the codon. R6 *pbp1a* sequence taken from (Martin et al., 1992a), accession number M90527.

5.2.3.2 Partial pbp2b gene sequence

Initially a 1.3 kb region of the *pbp2b* transpeptidase domain was sequenced from two isolates (98-537 and 99.539) using primers Pn2Bup and Pn2Bdown (Table 2.4).

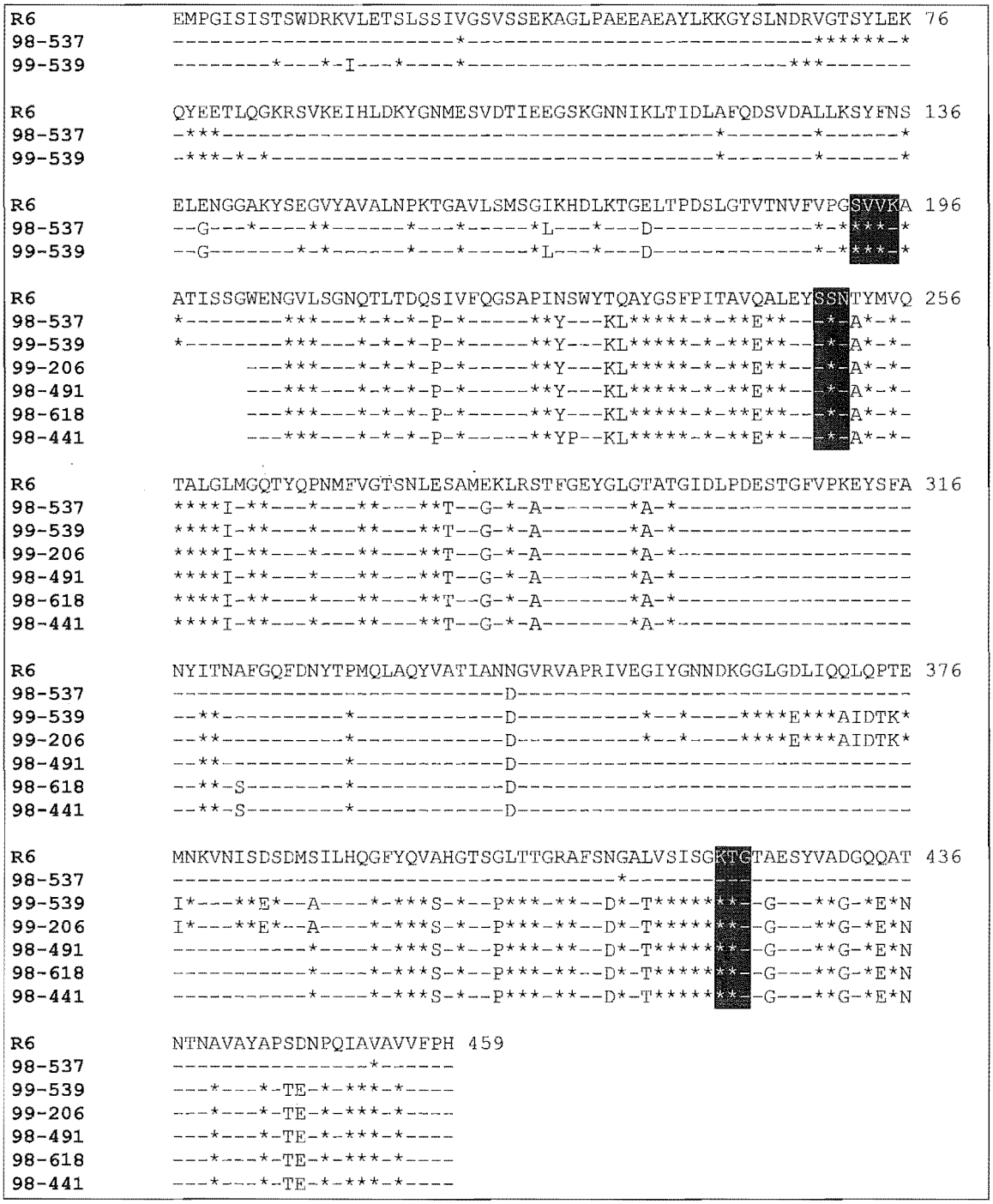

Figure 5.7 The predicted amino acid sequence *pbp2b* gene.
Shaded regions represent conserved motifs, dashes (-) indicate the amino acid sequence is identical to R6, asterisks (*) indicate a silent substitution in the codon. R6 *pbp2b* sequence taken from (Dowson et al., 1989b), accession number X13136.

The majority of the significant nucleotide substitutions occurred in a region between nucleotides 654 and 1341. This region began 66 nucleotides downstream of the sequence encoding the SER-VAL-VAL-LYS conserved motif. The area around this motif seems well conserved in several isolates examined (Smith and Klugman, 1995) and may not in itself play a large role in reducing the penicillin affinity of PBP2b. Subsequent DNA sequence was obtained from another four isolates targetting the region of maximum variability, nucleotides 609 – 1377, using primer pbp2b-seq and Pn2Bdown (primer sequences given in Table 2.4 and Table 2.5). Figure 5.7 shows the predicted amino acid, including codons with synonymous nucleotide substitutions.

The *pbp2b* genes of the six isolates contained between 58 (7.5%) and 134 (17.3%) nucleotide substitutions within the 773 bp region when compared to the *pbp2b* sequence of sensitive strain R6. The predicted amino acid sequence contained between 11 (4.3%) and 31 (12.1%) residues which differed from R6 (Figure 5.7). Within the 773 bp examined, three major regions of variability were evident (Figure 5.8). The first spanned nucleotides 616–858, and contained 54 (22.3%) nucleotide substitutions common to all six isolates. Thirty-six of the 54 substitutions were silent; the remaining 18 resulted in ten changes within the amino acid sequence. The second major region of divergence spanned nucleotides 1177–1365 and was common to all but one (98-537) isolate. This 188 bp region contained 48 (25.5%) nucleotide substitutions, of these 30 were silent, while the remaining 18 were responsible for ten changes in the predicted amino acid sequence. Isolate 98-537 did not contain this region of altered sequence and maintained 98.9% sequence identity with R6 over this region. A third region of variability was noted in the two most resistant strains (99-539 and 99-206). This is a 100 bp region spanning nucleotides 1064–1164. This region contained 31 (31%) nucleotide substitutions, of these 13 were silent and 16 were associated with nine amino acid substitutions.

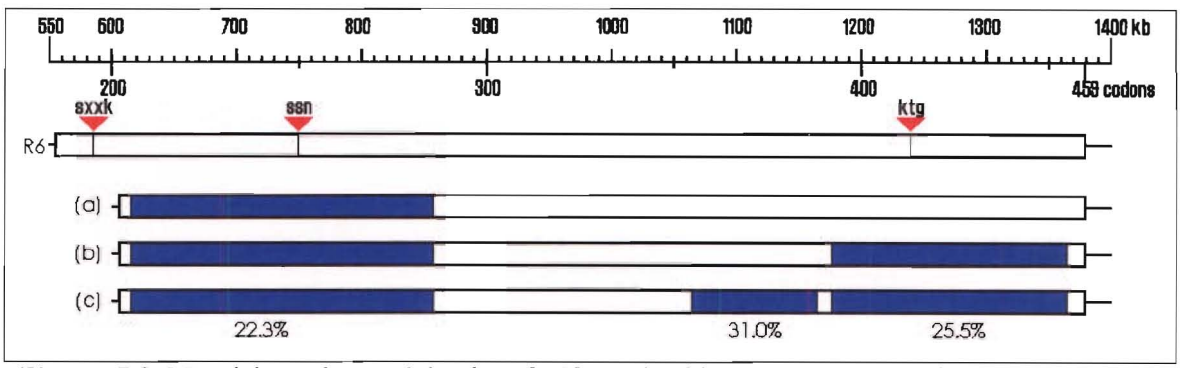

Figure 5.8 Mosaicism observed in the *pbp2b* nucleotide sequence. Shaded regions represent sequence divergent from that of sensitive strain R6,
a) strain 98-537, b) strains 98-491, 98-618 and 98-441, c) strains 99-539 and 99-206.

5.2.3.3 Partial pbp2x *gene sequence*

The nucleotide sequence of *pbp2x* was determined for eleven isolates of *S. pneumoniae* using primers listed in Table 2.4. A 1020 bp region was sequenced between nucleotides 1021 and 2040 (numbering based on (Laible et al., 1989)). Predicted amino acid sequences, including codons with synonymous nucleotide substitutions, are shown in Figure 5.9. The eleven isolates could be placed into one of three groups based on their *pbp2x* sequence. Group 1 contained five isolates (95-176, 99-539, 99-206, 99-205, and 98-560), each had 183 (17.9%) nucleotide substitutions resulting in 40 amino acid changes when compared to sensitive strain R6.

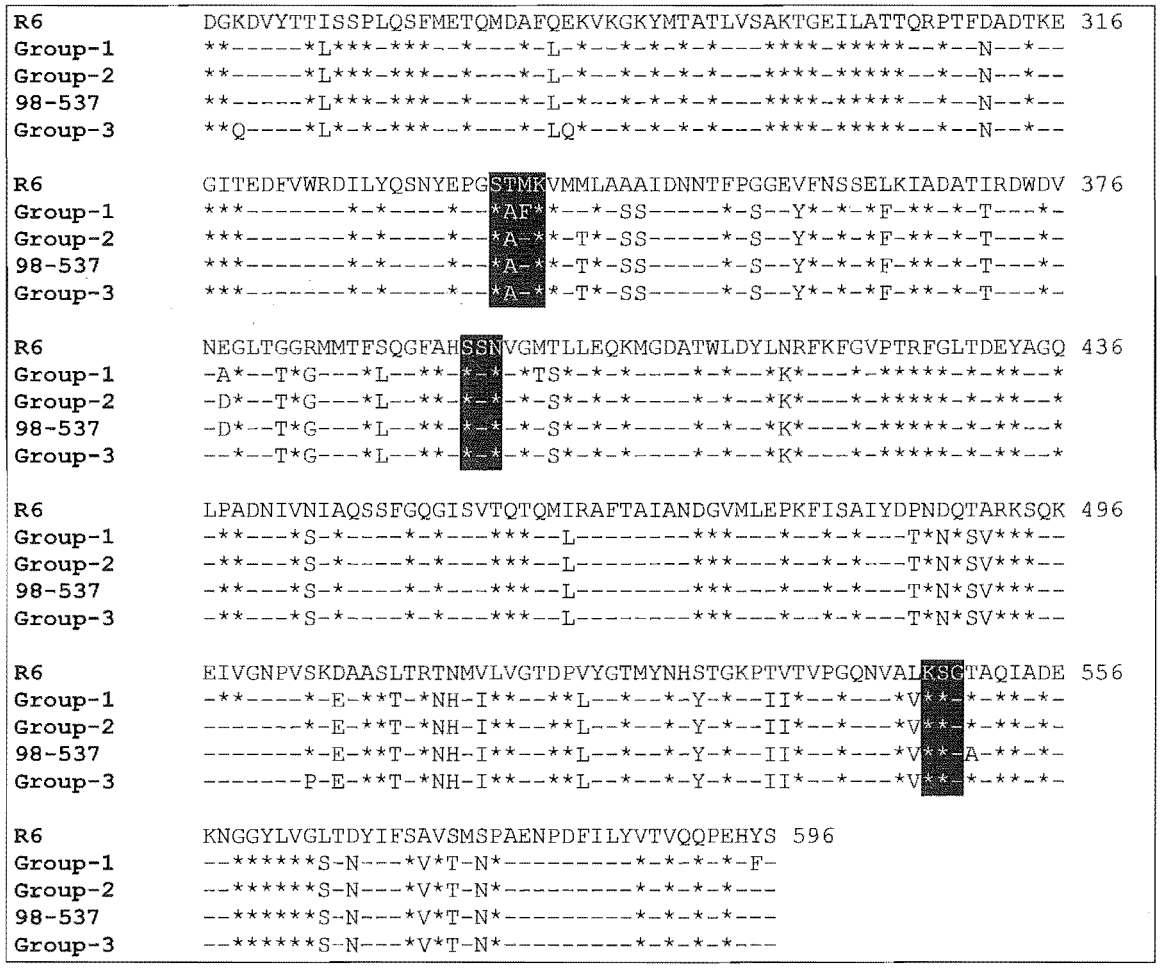

Figure 5.9 The predicted amino acid sequence *pbp2x* gene.
Shaded regions represent conserved motifs, dashes (-) indicate the amino acid sequence is identical to R6, asterisks (*) indicate a silent substitution in the codon. Group 1 consists of 6 isolates; 95-176, 99-539, 99-206, 99-205 and 98-560, Group 2 contains isolates 98-491, 98-120 and 97-816, Group 3 consists of isolates 98-618 and 98-441. R6 *pbp2x* sequence taken from (Laible et al., 1989), accession number X16367.

Group 2 contained three isolates (98-491, 98-120 and 97-816) that differ in their sequence of *pbp2x* by 180 (17.6%) nucleotides when compared to strain R6. Of the 180 nucleotide substitutions 129 (72%) were silent. The remaining 51 were associated with 38 amino acid substitutions. Isolate 98-537 was most similar in sequence to group 2, but had a single

A(1900)→G which resulted in an amino acid change of threonine to alanine immediately following the KSG conserved motif. For this reason this isolate is considered separately, although it is otherwise indistinguishable from group 2.

Group 3 contains two isolates (98-618 and 98-441). Both isolates differ from strain R6 at 181 (17.7%) nucleotides, of which 128 (70.7%) were silent. Group 3 isolates had 40 amino acid substitutions when compared to R6. Substitutions unique to group 3 include Lys(259) → Gln, Glu(282) → Gln and Ser(504) → Pro.

5.2.4 Protein labelling

Labeling of PBPs using Bocillin FL was performed as previously described (Zhao et al., 1999). Figure 5.10 shows Bocillin FL-labeled cell wall extracts from a penicillin-susceptible clinical strain. The sizes of the proteins binding the Bocillin FL are in congruence with the size which have been cited for high weight PBPS (between 92-78 kDa). Furthermore the resulting banding pattern resembles closely profiles documented for other sensitive pneumococcal isolates (Hakenbeck et al., 1980, Percheson and Bryan, 1980).

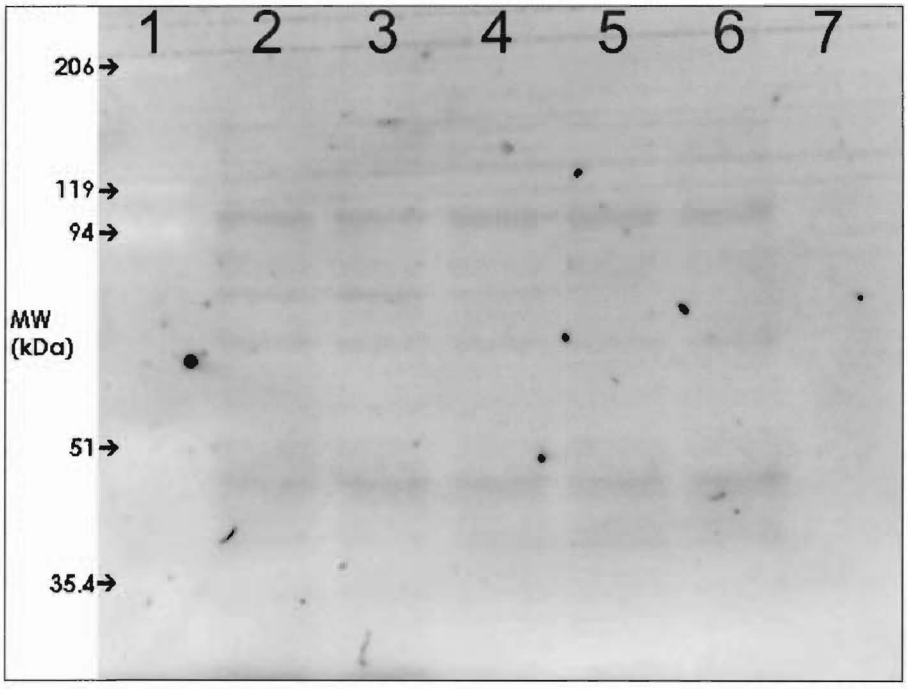

Figure 5.10 SDS-PAGE gel of Bocillin-FL labeled PBPs from isolate 96-079.
Lane 1 Molecular size marker, lanes 2-6 decreasing Bocillin-FL concentrations 10 μM, 5 μM, 1 μM, 0.5 μM, 0.25 μM respectively, lane 7 control containing no Bocillin-FL.

As was to be expected of PBPs from a sensitive isolate, they bind Bocillin FL at a low concentration. Furthermore, with decreasing concentration of Bocillin FL, less of the drug is

available to be bound by the proteins, and subsequently those with lower affinities are not labelled. In this instance the PBPs 1A, 1B and 3 appear to have the greatest affinity for Bocillin FL, while the affinities of PBP2A and 2B are less.

5.3 Discussion

5.3.1 Application of Bocillin FL

Bocillin FL holds great potential as a reagent to study the activities of penicillin-binding proteins without the need to employ radioactivity. Initial trials with this agent on penicillin-susceptible clinical isolates (such as 97-069 shown in Figure 5.10) showed great promise. The PBPs bound the reagent at the concentration suggested (Zhao et al., 1999), and produced interpretable profiles of PBPs. Unfortunately, these results could not be reproduced with penicillin-resistant isolates. Even at excessive drug concentrations, which should theoretically saturate all PBPs, the proteins could not be detected. Subsequent work with Bocillin FL was halted. It is possible the bulky attachment of the fluorescent component of Bocillin FL interferes with its interaction with resistant PBPs. If this is the case, radioactively labelled penicillin would be superior reagent for the analysis of resistant PBPs.

5.3.2 Interpretation of the nucleotide sequence of *pbp* genes

5.3.2.1 Interpretation of pbp1a *nucleotide sequence*

The *pbp1a* nucleotide sequences were essentially identical for all four isolates sequenced. This is a notable finding as PBP1A is one of the PBPs most implicated in resistance to cephalosporins (Munoz et al., 1992), and isolates with maximum variation in cefotaxime MIC were selected for DNA sequence analysis (range 0.5 – 16 μg/ml).

Blocks of mosaic sequence were not observed in the partial nucleotide sequence of *pbp1a*, rather the first 970 bp of sequence contained multiple substitutions, resulting in 42 changes in the predicted amino acid sequence. The remaining 150 bps sequenced did not differ in sequence from the corresponding sequence of pneumococcal isolate R6. This structure is not dissimilar to that which has been described for mosaic *pbp1a* genes previously (Coffey et al., 1995a). The amino acid sequence of *pbp1a* of the New Zealand 19F clone resembles that of two classes of PBP described previously (Asahi and Ubukata, 1998). Residues 320 – 505 match exactly with isolate 14/Z42 (group IV), and residues 506 – 675 match with isolate 2/H26 (group III) in all but two residues (Ala550 and Ser657) (Asahi and Ubukata, 1998). The two isolates are quite different, suggesting that at least two insertions from different donors may have occurred in the New Zealand clone. Isolate 14/Z42 is a serotype 14 with penicillin and cefotaxime MICs of

0.125 and 0.25 μg/ml respectively; and isolate 2/H26 is a serogroup 19 isolate with penicillin and cefotaxime MICs of 2 and 0.5 μg/ml respectively.

All four isolates sequenced have the Thr-371→Ser substitution within the Ser-X-X-Lys conserved motif of PBP1A. This change is synonymous with the Thr-338→Ala change within the Ser-X-X-Lys motif of resistant PBP2x. It does not seem unreasonable that the structural implications of this substitution in PBP2x also hold true in PBP1a (see section 5.3.2.3). This alteration was noted within two isolates previously described, and thought to contribute to penicillin resistance (Asahi and Ubukata, 1998), as well as in a highly resistant *S. mitis* isolate (Hakenbeck et al., 1998).

5.3.2.2 Interpretation of pbp2b *nucleotide sequence*

Analysis of the partial *pbp2b* gene sequence revealed three regions of divergence from the sensitive strain R6 (Figure 5.8). The first region of divergence resulted in 11 amino acid substitutions, and was associated with a minimum penicillin MIC of 0.5 μg/ml. This region of ~250bp falls in the centre of the transpeptidase encoding region of *pbp2b* and is flanked by two of the three conserved amino acid motifs common to all penicilloyl transferases (Ghuysen, 1991); the Ser-X-X-Lys tetrad (residues 192-195) and the Ser-X-Asn triad (residues 249 – 251). The amino acid substitutions observed within this block also coincide with what has been previously described as a "class B" *pbp2b* gene (Dowson et al., 1989a). The resistant class B *pbp2b* genes differed from their homolog in the sensitive strain R6 by 75 nucleotide substitutions. All but one of these substitutions occurs within the first 331 codons, resulting in 14 amino acid changes. This is very similar to what was observed within the *pbp2b* gene of isolate 98-537. Dowson and colleagues described isolates recovered as early as 1984 (penicillin MICs 2 μg/ml) that were found to contain the class B *pbp2b* gene. This suggests that the original transformation event that led to this particular mosaic *pbp2b* gene may have occurred up to two decades ago.

The observations noted in class B resistant *pbp2b* genes, were identical to those described as 'profile 4' by Smith and Klugman (1995). The four 'profile 4' isolates were serotype 6 (1) and 19 (3), and had penicillin MICs of 4 (2) and 0.25 (2) μg/ml. This profile was one of five described by Smith and Klugman, yet it was common to all six isolates examined in the current study. It is suggested that these changes resulted in, at least, an increase to an intermediate level of penicillin resistance (Smith and Klugman, 1995). The current study supports this observation, as one isolate (98-537) where only the first region diverged from R6 (Figure 5.8), had an

intermediate penicillin resistance (0.5 μg/ml). The other five isolates had additional regions of divergence (block 2 and 3) and correspondingly greater penicillin MICs. It has been suggested that this area of *pbp2b* forms part of the penicillin-binding site that interacts with the R1 side group of penicillin. In particular, the importance of the alanine for threonine-252 has been noted (Dowson et al., 1989b). This substitution occurs adjacent to the Ser-X-Asn motif. Based on comparison with class A β-lactamases, it is proposed the threonine residue forms a hydrogen bond with the carbonyl group of the R1 side chain of penicillin. The substitution of threonine with alanine, removes the hydroxyl group previously available to form the hydrogen bond.

Group	Amino acid substitutions	Isolates	Pen MIC	Ceft MIC
Group A (12 subs.)	S218P, N228Y, T232K, Q233L, Q244E, T252A, L261I, S279T, E282G, S286A, T295A, N334D	98-537	0.5	16
Group B (10 subs.)	A398S, G403P, N412D, L415T, A425G, D431G, Q434E, T436N, S446T, D447E	98-441	4	1
		98-491	2	1
		98-618	2	1
Group C (9 subs.)	D367E, Q371A, L372I, Q373D, P374T, T375K, M377I, D384E, S388A	99-206	4	8
		99-539	4	8

Table 5.4 Amino acid substitutions associated with major divergent blocks in *pbp2b* of MRP group 1 isolates.

The Thr-252→Ala substitution is one of only three from this block that are consistently associated with reduced penicillin-susceptibility. Glu-282→Gly is also a common substitution, the significance of which has not yet been elucidated. A third substitution Asn334→Asp, although lying outside the region of divergence, may be significant as it is found associated with many resistance pneumococcal isolates (Smith and Klugman, 1995).

The second block of amino acid substitutions (Figure 5.8) occurred between residues 398 and 447. This region also contains a conserved amino acid motif; the Lys-Thr-Gly triad (residues 421-423). This block of altered amino acid sequence was found in five of the six isolates sequenced, and was associated with increased penicillin MICs (2-4 μg/ml). The second block contained ten amino acid substitutions (Table 5.4). Nine of the ten substitutions had been observed previously by Smith & Klugman (1995) in a "profile 4" isolate. Of the four "profile 4" isolates described by Smith & Klugman, three displayed high amino acid sequence identity to the originally described resistant "class B" *pbp2b* sequence (Dowson et al., 1989a).

5.3.2.3 Interpretation of pbp2x *nucleotide sequence*

The gene encoding PBP2x of *S. pneumoniae* R6 had its DNA sequence determined in 1989 (Laible et al., 1989). Also sequenced was a laboratory mutant C506, in which three amino acid susbstitutions were observed; Met-289→Thr, Gly-597→Asp, and Gly-601→Val. These three alterations occurred at either end of the transpeptidase region of PBP2x, and their contributions to resistance could not be fully elucidated (Laible et al., 1989). The latter two substitutions lie outside the region sequenced in this study. Analysis of crystal structure data has revealed these residues compromise part of an α-helix and are quite distal to the active site (Gordon et al., 2000). Consequently they may not interact directly with β-lactam antibiotics. Sequence analysis of laboratory mutants created by PCR-derived mutagenesis identified the substitution of Thr-550→Ala that imparted a fourfold increase in resistance to cefotaxime (Grebe and Hakenbeck, 1996). This alteration was observed in only one of the eleven isolates sequenced in this study; isolate 98-537. Interestingly isolate 98-537, while having a high resistance to cefotaxime, has an uncharacteristically low penicillin MIC of 0.5 μg/ml. The Thr-550→Ala mutant described although having an increased cefotaxime MIC, also had a decreased oxacillin MIC (Grebe and Hakenbeck, 1996). This suggests a possible mechanism of increased cefotaxime-resistance at the expense of increased sensitivity to penicillin. This is demonstrated further by Sifaoui and colleagues (Sifaoui et al., 1996), who showed that the single amino acid substitution Thr-550→Ala was responsible for an increase in cefpodoxime resistance while becoming more sensitive to amoxicillin.

Thr-550 lies within a β-sheet (β3) within 6.5Å of the active site serine residue (Gordon et al., 2000). Thr-550 binds the carboxylate group of the cephalosporin ring via the hydroxyl side chain O^{χ}. The α-amide group also H-bonds with the cephalosporins. The mutation Thr-550→Ala reduces enzyme efficiency for 2nd and 3rd generation cephalosporins. Site directed mutagenesis of Thr-550→Ala in strain R6 showed a 90% reduction of acetylation efficiency by cefotaxime, while penicillin G was unaffected (Mouz et al., 1999). This change was accompanied by a 46% decrease in hydrolytic activity of the mutant on substrate analogue of cell wall peptides (Mouz et al., 1999).

In the current study, the predicted amino acid sequence of PBP2X was organised into three main groups on the basis of sequence identity. Group I contained highly resistant isolates with penicillin and cefotaxime MICs in the range of 4-8 and 2-16 μg/ml, respectively. The predicted amino acid sequences of group I isolates have sequence similarities with previously described group V isolates of a study performed by Asahi and colleagues (Asahi et al., 1999). This is

particularly evident around the conserved amino acid motifs, including two substitutions within the STMK motif; a Thr-338→Ala, and a Met-339→Phe; and a Lys-546→Val substitution just prior to the KSG motif. The isolates with all three of these substitutions tended to have high cefotaxime MICs (2-8 μg/ml), which is in agreement with the current study. Isolates which lacked the Met-339→Phe substitution (Group IV) tended to have lower cefotaxime MICs (0.5-1 μg/ml) (Asahi et al., 1999), which again is in agreement with isolates of groups II and III of the current study.

Recently the crystal structure of a resistant PBP2X enzyme was described (Dessen et al., 2001). The enzyme was derived from a clinical isolate, Sp328, which contains 92 mutations when compared to the sensitive form derived from R6. It was shown that the Thr-338→Ala substitution within the STMK conserved motif weakens the local hydrogen bonding network within the active site. Other substitutions of interest include the Ser-389→Leu and Asn-514→His, (both observed in all eleven isolates of the current study) which open the active site. This may not be involved in the resistance mechanism proper, but alters the catalytic function of the enzymes allowing abnormal branched peptides to be used as substrates.

5.3.2.4 Overall interpretation of pbp *gene nucleotide sequences*

Much information can be derived from examination of DNA sequence, and the predicted amino acid sequence derived from it. Such examinations are enhanced if other information, such as crystallographic structure data is available. In the current study, it was shown that although a seemingly homogenous clone, the Taiwan^{19F}-14 clone has a lot of variability in its β-lactam MICs, which at the molecular level is imparted by several different mosaic *pbp* genes. It seems also that the *pbp* genes of the Taiwan^{19F}-14 clone have been acquired by horizontal gene transfer from other resistant *S. pneumoniae* isolates. This is supported by the observation that all mosaic *pbp* genes described were very close, often identical, to those described in the literature.

Chapter 6

Macrolide Resistance Mechanisms

6.1 Introduction

The macrolide group of antibiotics are useful broad-spectrum agents that have proved effective in the treatment of upper and lower respiratory tract infections, being particularly effective against streptococcal and mycoplasmal infections. This class of antibiotic, typified by erythromycin, is especially useful in the treatment of persons allergic to penicillin. Macrolides were originally isolated from *Streptomyces* species, and acquired their name from their chemical structure, a macrocyclic lactone ring to which various amino sugars are attached. The most clinically important macrolides are the 14-, 15- and 16-membered ring compounds, which consist of natural products and semisynthetic derivatives. Macrolides owe their activity to their capacity to inhibit protein synthesis. Macrolides reversibly bind the 50S ribosomal subunit, inhibiting the ribosomal transpeptidation/translocation process, causing premature detachment of incomplete peptide chains (Retsema and Fu, 2001).

There are currently three recognised mechanisms of resistance to macrolide antibiotics; target modification, inactivation, and efflux (Pechere, 2001). A large number of resistance determinants that confer these macrolide resistance mechanisms have been identified (Roberts et al., 1999). The two most common mechanisms of macrolide resistance reported in *S. pneumoniae* are target modification and drug efflux, mediated by the *erm* and *mef* genes respectively (Pechere, 2001).

The *erm*(B) gene (previously *ermAM*) product methylates a specific adenine residue (A2058) within the 23S rRNA (Weisblum, 1995). This modification conveys cross-resistance to macrolides, lincosamides and streptogramin B compounds (MLS_B phenotype) and is also associated with high-level resistance (≥64 μg/ml).

The *mef*(A) gene (previously *mefE*, but reclassified due to high sequence identity with *S. pyogenes mefA* (Roberts et al., 1999)) encodes an efflux pump, which is specific only for 14-

and 15- membered macrolides (M phenotype) (Tait-Kamradt et al., 1997). The macrolide efflux system was first reported in *S. pneumoniae* and *S. pyogenes* in 1996 (Sutcliffe et al., 1996b).

The prevalence of either mechanism tends to be specific to certain geographic locations. In the United States, the M phenotype is more prevalent (Shortridge et al., 1999), while in Europe the MLS_B phenotype dominates (Schmitz et al., 2001). Although pneumococcal resistance to erythromycin has been documented in New Zealand (Brett et al., 1999), no studies on the mechanisms of resistance have been performed. The current chapter describes the prevalence of two macrolide resistance determinants (*erm*(A) and *mef*(B)) in New Zealand, as assessed by PCR analysis.

6.2 Results

6.2.1 Erythromycin MIC determination

Of the 200 clinical isolates collected from Christchurch during 1997-2001, 150 isolates exhibiting resistance to erythromycin were randomly selected. These isolates had been previously screened for erythromycin resistance using a disc diffusion assay. Erythromycin MICs were determined by agar dilution testing. Of the 150 erythromycin-resistant isolates, 141 (94.0%) had high-level erythromycin resistance (MIC > 256 μg/ml). The remaining 9 (6.0%) isolates had MICs between 2-8 μg/ml. Erythromycin resistance was typically associated with resistance to other antimicrobial agents; one hundred and forty-two (94.7%) isolates were also resistant to co-trimoxazole, 137 (91.3%) were resistant to tetracycline and 26 (17.3%) were resistant to chloramphenicol.

6.2.2 PCR detections of macrolide resistance genes

PCR detection of the macrolide resistance determinants was performed on all 150 isolates (Figure 6.1). The *mef*(A) gene was detected in 105 (70.0%) isolates and the *erm*(B) gene in 142 (94.7%) isolates (Table 6.1). Surprisingly, both the *mef*(A) and *erm*(B) genes were detected in 97 (64.7%) isolates. In all but one instance, when both genes were detected in the same isolate, its corresponding erythromycin MIC was >256 μg/ml. The high-level erythromycin resistance observed was presumably imparted by a functional *erm*(B) gene.

The *erm*(B) PCR primers also generated a "ghost" band, approximately 550 bp in size (Figure 6.1). This band was consistently associated with *erm*(B) positive PCR product, and absent when the PCR was negative. Given the consistent association with the presence of the *erm*(B) PCR product proper, it seems likely this secondary product may be due binding of one

of the primer pair elsewhere within the *erm*(B) gene. Consequently, determination of the identity of this product was not pursued.

Resistance determinant detected by PCR	Number (%) of isolates falling into MIC range	
	1.0 – 8.0 μg/ml	≥256 μg/ml
erm(B) determinant only	0	45 (30.0)
mef(A) determinant only	8 (5.3)	0
Both *erm*(B) and *mef*(A) determinants	1 (0.7)	96 (64.0)

Table 6.1 Erythromycin resistance determinants detected in 150 isolates of *S. pneumoniae* and their corresponding erythromycin MIC's.

Of the 150 isolates tested for the presence of either resistance gene, 145 (96.6%) belonged to one of five MRPs. The association of either the *erm*(B) gene or *mef*(A) gene with each of the five MRPs is shown in Table 6.2. Of the 97 isolates containing both resistance genes, 93 (95%) belonged to MRP group 1; the profile of New Zealand variant of the Taiwan^{19F}-14 clone (Korea^{19F}-14 clone). Macro restriction profile groups 9 and 11 were associated only with the *erm*(B) gene. Similarly MRP group 2 was associated with both genes, although predominantly (71%) the *erm*(B) gene. MRP group 10 was associated with either gene, although the *mef*(A) was more common (75%).

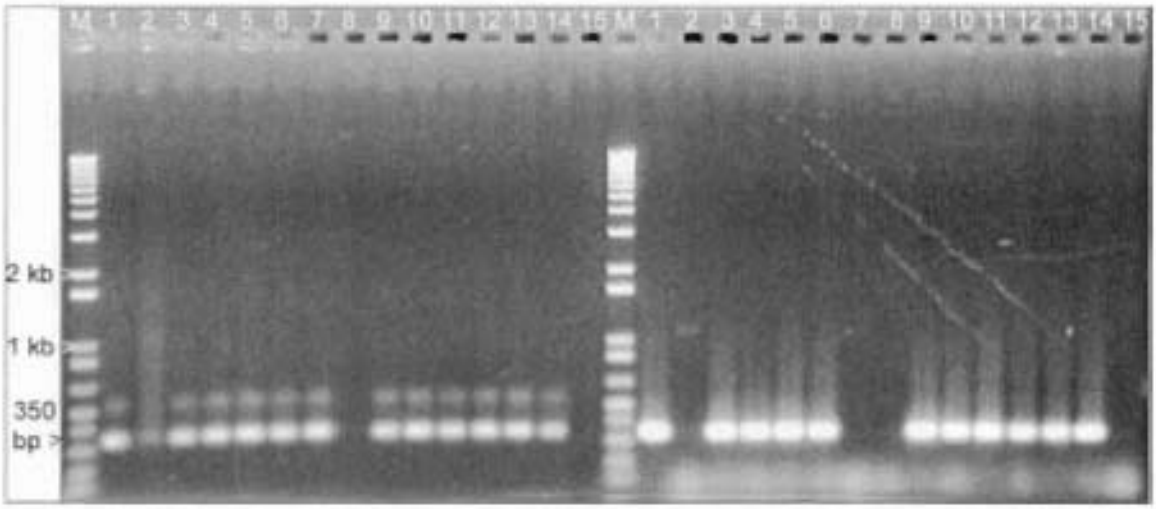

Figure 6.1 Example of *erm*(B) (left) and *mef*(A) (right) gene PCR detection.
Lanes order (nb lane order identical for both *erm*(B) and *mef*(A)); M) Marker, 1 kb+ ladder (Gibco) 1) isolate SP039, 2) isolate SP043, 3) isolate SP051, 4) isolate SP057, 5) isolate SP059, 6) isolate SP061, 7) isolate SP078, 8) isolate SP089, 9) isolate SP110, 10) isolate SP115, 11) isolate SP119, 12) isolate SP133, 13) isolate SP138, 14) isolate SP145, 15) ATCC 49619 (-ve control)

Macro-restriction group	Number analysed	Positive for *erm*(B) gene only (%)	Positive for *mef*(A) gene only (%)	Positive for both *erm*(B) and *mef*(A) gene (%)
1	95	0	2 (2)	93 (98)
2	24	17 (71)	4 (17)	3 (12)
9	4	4 (100)	0	0
10	4	1 (25)	3 (75)	0
11	18	18 (100)	0	0

Table 6.2 Association of *erm*(B) and *mef*(A) genes with four different macro restriction groups.

6.2.3 DNA sequence analysis of macrolide resistance genes

To verify the identity of the amplicons generated from the Korea^{19F}-14 clone the nucleotide sequence of PCR amplicons was determined. PCR amplicons from isolate 99-539 were used as templates for DNA sequencing. The resulting nucleotide sequences of the *erm*(B) and *mef*(A) genes had 98% and 100% sequence identity respectively, to the synonymous genes in the Genbank database.

6.2.4 Southern blot detection of macrolide resistance genes

To verify the presence of these genes in the genome, Southern hybridisation experiments were performed on *Sma*I-digested genomic DNA separated by PFGE (Figure 6.2). Probes were synthesized using PCR. The same primers used for identification of the *erm* and *mef* genes were used to generate the probes. In each case, probes for both genes hybridised with the same 120 kb DNA fragment of the Taiwan^{19F}-14 genomic digest (isolate 99-539). Isolates in which only the *erm* gene had been detected by PCR hybridised only with the *erm* probe. The *erm* probe bound to the same 388 kb DNA fragment in the three isolates of MRP 2. This is consistent with Spain^{23F}-1 isolates from Italy (Marchese et al., 1998). Isolate Sp017 with low level macrolide resistance (MIC 4 μg/ml) hybridised only with the *mef* probe, consistent with the results of PCR detection. The size of the restriction fragment to which respective probes hybridised is given in Table 6.3. Three isolates were included in the Southern hybridisation analysis serving as negative controls, isolates Sp046, Sp081 and strain RX1.

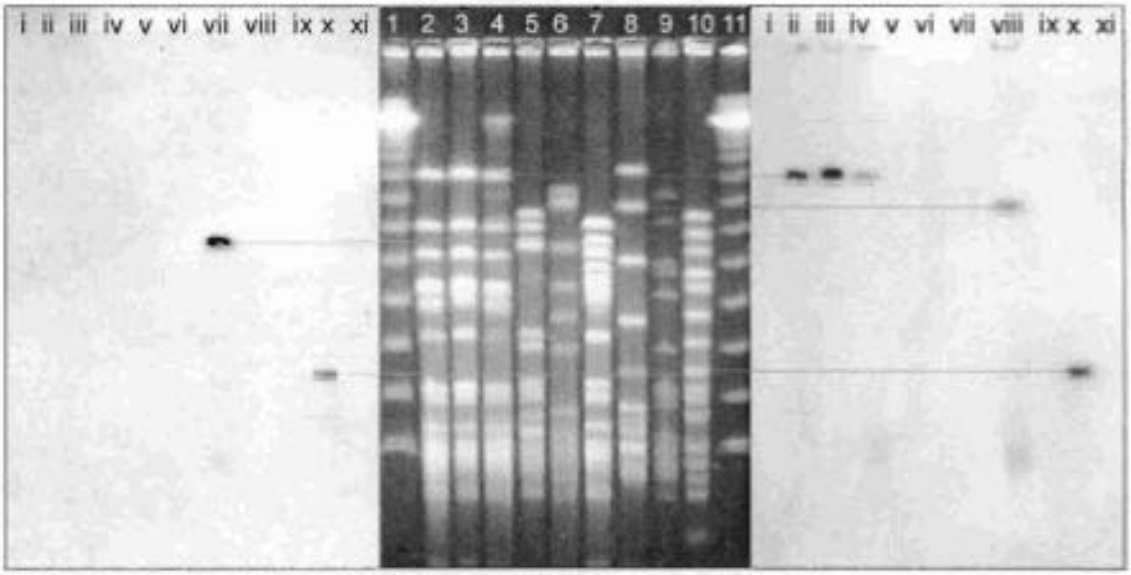

Figure 6.2 Detection of erythromycin resistance determinants in *Sma*I genomic digest by Southern blot analysis.

Centre panel – chromosomal DNA digested with *Sma*I and separated by PFGE, left panel – detection of macrolide efflux genes with *mef*(A) gene probe, right panel – detection of ribosome methylation genes with *erm*(B) gene probe. Lanes 1) Lambda ladder, 2) isolate Sp035, 3) isolate Sp105, 4) isolate Sp020, 5) isolate Sp046, 6) isolate Sp081, 7) isolate Sp017, 8) isolate Sp058, 9) strain RX1, 10) isolate 99-539, 11) Lambda ladder. Lanes i) to xi) have identical order.

To further investigate whether other New Zealand variants of the Taiwan^{19F}-14 clone contained the two erythromycin resistance determinants, Southern hybridisation with the same probes was performed on another *Sma*I genomic digest of representative isolates (Figure 6.3).

Isolate	Macro-restriction profile	Size of fragment (kb) binding *mef* probe	Size of fragment (kb) binding *erm* probe
Sp035	2A	--	388
Sp105	2A	--	388
Sp020	2A	--	388
Sp017	10A	266	--
Sp058	11D	--	340
99-539	1A	120	120

Table 6.3 Size of *Sma*I restriction fragment which was hybridised by either *erm* or *mef* probe.

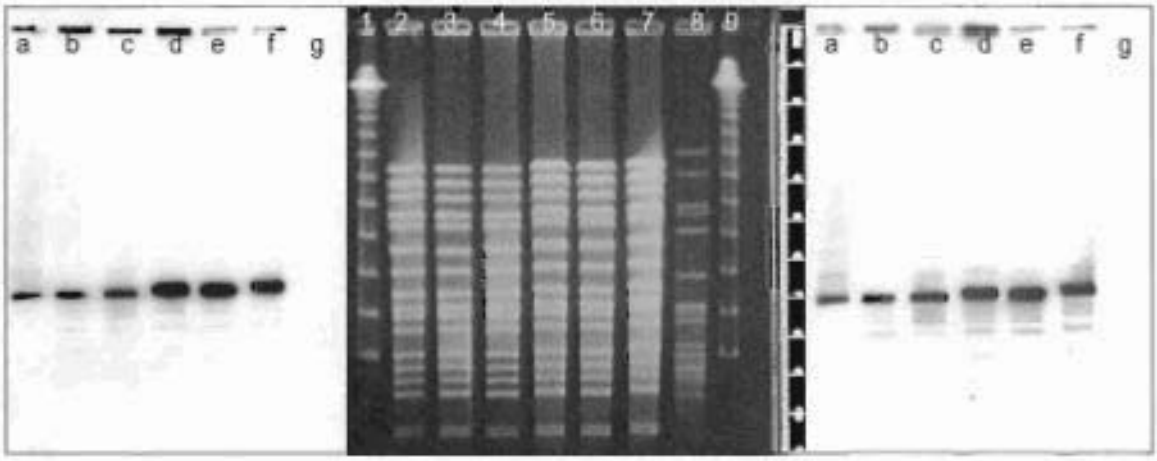

Figure 6.3 Detection of erythromycin resistance determinants by Southern blot analysis in *Sma*I genomic digests of Taiwan^{19F}-14 isolates.
Centre panel – chromosomal DNA digested with *Sma*I and separated by PFGE, left panel – detection of macrolide efflux genes with *mef*(A) gene probe, right panel – detection of ribosome methylation genes with *erm*(B) gene probe. Lanes 1) Lambda ladder, 2) isolate Sp035, 3) isolate Sp105, 4) isolate Sp020, 5) isolate Sp046, 6) isolate Sp081, 7) isolate Sp017, 8) isolate Sp058, 9) strain Rx1, 10) isolate AR99-539, 9) Lambda ladder. Lanes a) to g) correspond to lanes 2-8, respectively.

6.3 Discussion

Typically, erythromycin-resistant pneumococci from any given geographic location will possess only one of the two most commonly described resistance mechanisms. In the United States the *mef*(A) gene is more dominant, being identified in 61% of 114 macrolide resistant isolates examined by Shortridge and colleagues (1999). By contrast, screening European countries such as France and Italy have found the *erm*(B) gene in 81.4% and 84.1% of erythromycin-resistant isolates, respectively (Schmitz et al., 2001). In Christchurch, the predominant macrolide resistance genotype is mediated by both *erm*(B) and *mef*(A) genes. This genotype was identified in 62.1% of the Christchurch isolates examined in this study. Given that other major centres in New Zealand have comparable rates of erythromycin resistance to those seen in Christchurch (Brett et al., 1999), it can be assumed this observation can be applied to the country as a whole.

Although an uncommon genotype, a recent report from South Africa found 36 of 118 (30.5%) erythromycin-resistant isolates tested contained both *erm*(B) and *mef*(A) genes by PCR (McGee et al., 2001a). DNA fingerprinting of these 36 serotype 19F isolates identified that 30 isolates belonged to a single clone. Based on the phenotypic evidence presented, as well as the macro-restriction profiles associated with the South African isolates, it seems likely that the South African isolates are related to those from New Zealand, and ultimately, both derived from the Taiwan^{19F}-14 clone. This is of note, because it would verify the movement of the Taiwan^{19F}-14 clone to another continent. This clone has previously been identified in Europe (Enright and

Spratt, 1998), and the current study has shown the Taiwan^{19F}-14 to be the dominant resistant clone in New Zealand. However, both the South African and New Zealand variants of this clone differ from the Taiwan^{19F}-14 clone in that they have high level (MIC >256 μg/ml) erythromycin resistance, while the Taiwan^{19F}-14 clone is reported as containing only the *mef*(A) gene and having low level resistance (MIC 1.5-8.0 μg/ml) (Shi et al., 1998, McGee et al., 2001b). Only two of the Taiwan^{19F}-14 isolates in this study have this genotype (Table 6.2)

Isolate Sp413 had a low-level of erythromycin resistance although both resistance genes were detected; it was also susceptible to clindamycin when tested by disc diffusion. Low-level erythromycin resistance, and susceptibility to clindamycin, are characteristics of the M phenotype (Sutcliffe et al., 1996b, Tait-Kamradt et al., 1997). Presumably, therefore, isolate Sp413 contained a defective *erm*(B) gene, which still permitted its detection by PCR, and a low level of resistance mediated by products of a functional *mef*(A) gene.

The presence of both macrolide resistance genes mapping to the same ~5% of the pneumococcal genome raises the possibility the two genes may be linked. This could potentially be explained by an apparent pre-selection of pneumococcal transposons to interrupt other transposons and form composites. For example, conjugative transposon Tn*5251* (of the Tn*916* family) was found integrated into Tn*5252* to form the composite element Tn*5253* (Provvedi et al., 1996). Transposon Tn*917* was found integrated in Tn*3872* (McDougal et al., 1998). Furthermore, in the recently sequenced pneumococcal genome (Dopazo et al., 2001), a locus was identified which appeared to have been the result of three separate transposition events. Both *erm*(B) and *mef*(A) have been identified on transposons in *S. pneumoniae*. The *erm*(B) gene is typically carried on a conjugative transposon Tn*1545*; a 25.3 kb element that imparts resistance kanamycin and related aminoglycosides, tetracycline, and the MLS_B antibiotics (Courvalin and Carlier, 1986). The *mef*(A) gene has recently been identified on a transposon, Tn*1207.1* (Santagati et al., 2000); a 7244 bp mobile element. Therefore, a potential explanation for the relatively close proximity of *erm*(B) and *mef*(A) may be that they have formed a composite transposon.

This study focused on the two most common erythromycin resistance determinants, as described in the literature. That does not preclude the existence of other resistance mechanisms in this population that may have been overlooked. Several other resistance determinants have recently been described in *S. pneumoniae*. An unusual derivative of the M phenotype, imparted by a mutation in the 23S rRNA, provides resistance to 16-membered macrolides (Depardieu and

Courvalin, 2001). The $A_{2062}C$ mutation was present in all four copies of the 23S rRNA of isolate BM4455, and was associated high MICs (512 μg/ml) for 16-membered macrolides, such as spiramycin, but was sensitive to 14-membered (e.g., erythromycin) and 15-membered (e.g., azithromycin) macrolides. This isolate was also sensitive to lincosamide and streptogramin antibiotics. Other mechanisms of resistance have been observed imparting ML and MS_B phenotypes (Tait-Kamradt et al., 2000a). The former phenotype is associated with an $A_{2059}G$ mutation in the 23S rRNA, and the latter with a three amino acid substitution ($_{69}GTG_{71}$ to TPS) in the ribosomal protein L4. Serial passage of *S. pneumoniae* in the presence of azithromycin has produced similar mutations in the 23S rRNA *in vitro* (Tait-Kamradt et al., 2000b). An *erm*(A) methylase (previously *erm*(TR) of *S. pyogenes*) gene has been identified in *S. pneumoniae* (Syrogiannopoulos et al., 2001). The *erm*(A) determinant imparts low-level resistance to 14- and 15-membered macrolides, is inducibly resistant to clindamycin, and susceptible to streptogramin B.

The results from this study show that the most predominant erythromycin resistance genotype in New Zealand is *erm*(B) *mef*(A). This combination of genes imparts a phenotype essentially identical to that of *erm*(B) alone. The majority of *erm*(B) *mef*(A) strains in New Zealand appear to belong to a multi-resistant serotype 19F clone, which has much in common with a recently multi-resistant 19F clone recently described in South Africa (McGee et al., 2001a). Assuming these strains are identical, this would support the global spread of this clone.

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Chapter 7

Discussion

7.1 Summary of Results

The current study was under taken with the premise of investigating the population structure of antibiotic-resistant *S. pneumoniae* in New Zealand. The prevalence of penicillin resistance in New Zealand has been increasing steadily every year over the past decade, in both invasive and non-invasive isolates. Despite this increase, investigation into the population structure of the organisms responsible for such a dramatic increase has been at best superficial. The lack of enquiry into the nature of the spread of resistant pneumococci limits any possibility of controlling the further spread, and understanding how it originated. To this end, a detailed molecular study of resistant isolates from a defined region (Christchurch) was performed. Two hundred isolates were recovered over a five-year period, from 1997-2001 and subjected to genotypic methods of strain identification. Christchurch served as a good population base for acquisition and testing of resistant pneumococci, having rates of resistance comparable, if not higher, than other centres in the country.

Of the 200, 97 (48.5%) were found to have related *Sma*I DNA macro restriction profiles. This observation supported the first of our hypotheses, that a penicillin-resistant pneumococcal was responsible for the increased prevalence in resistance observed in Christchurch. Further investigation of this group of isolates revealed many insights. The clone appeared to be genetically homogenous as assessed by *Sma*I digestion and separation of genomic DNA by PFGE. To investigate the homogeneity of the strain further, a second enzyme was employed, *Apa*I. Genomic digests with *Apa*I proved to be just as stable as those performed with *Sma*I. Analysis of the genes encoding penicillin-binding proteins (*pbp2b* and *pbp2x*) in this clone also showed they were conserved among isolates, although a slight degree of variation was noted.

Phenotypically, members of the predominant New Zealand clone were also homogenous. All isolates belonging to this clonal group for which serotype data was available indicated the clone expressed capsular type 19F. Furthermore all members of the clone also displayed identical antibiotic resistance phenotypes; resistance to co-trimoxazole, erythromycin and tetracycline,

and susceptibility to chloramphenicol and vancomycin. Interestingly, phenotypic variability was observed among the members of the predominant New Zealand 19F clone as variations in susceptibility to β-lactam antibiotics. The range of MIC for penicillin and cefotaxime was 1-8 μg/ml and 0.5-16 μg/ml respectively. This observation prompted interest in this clone not only from a epidemiological view, but also with regard to the clones variable β-lactam resistance.

The major New Zealand clone looked unlike any other international clone described at the time. However, the rapid increased in prevalence of such a genetically homogenous clone suggested that it may have, in fact, been imported into New Zealand and successfully disseminated. To test the hypothesis that the prominent New Zealand resistant pneumococcal clone had been imported into the country from an exogenous source, we applied the recently described technique, multilocus sequence typing. Furthermore, to verify that the clone was not limited to Christchurch, MLST was applied to a group of isolates recovered throughout New Zealand.

Analysis by MLST identified the major multi-drug resistant 19F clone to be indistinguishable from a 19F clone first identified in Taiwan. The PMEN designation for this clone is Taiwan^{19F}-14. Most of the New Zealand examples of the Taiwan^{19F}-14 clone differed from the Taiwan^{19F}-14 proper, in that they had an altered *aroE* allele. This alteration gave a sequence type that belonged to the Korean variant of the Taiwan^{19F}-14 clone.

7.2 Future research

The current study has provided evidence that supports the hypothesis that penicillin-resistant pneumococci were imported into New Zealand. Furthermore, Asia was identified as a likely source of incoming resistant organisms. Many of the newly emerging resistant isolates have been identified as having novel resistance phenotypes and genotypes. Consequently the current thesis immediately suggests two main avenues for further research:

a) The continued molecular surveillance of antibiotic-resistant bacteria in the Asia/Pacific region to identify and monitor emerging resistance trends.
b) The further molecular characterisation of antibiotic resistance phenotypes.

The first of these two points has obvious public health benefits. Although the WHO currently monitors levels of antibiotic resistance in the Asia/Pacific region, it does not provide any information with regard to population structure. Concerted collaborations between laboratories from this part of the world could strengthen these reports. Of particular importance is molecular

data for strain identification to monitor epidemic clones, and the significance they have on resistance rates. The second point, molecular characterisation of antibiotic resistance phenotypes, is important in the identification of new resistance determinants. Understanding of resistance mechanisms is important for the design of new antibiotics, and to make better use of existing agents.

7.2.1 On-going molecular epidemiology

This work highlights the need for greater surveillance of movement of pathogens and antimicrobial resistance in the Pacific region. Parts of Asia have reported extremely high rates of pneumococcal resistance. With New Zealand's status as a "nation of travellers" there is ample opportunity for persons entering the country to unwittingly bring in new pathogens, or antibiotic-resistant variants of existing pathogens. This study illustrates the use of molecular methods of strain identification to characterise a resistant population, and the use of that knowledge to trace the resistant organisms back to their probable origin. Further work in this field is certainly warranted to monitor movement of new and existing pathogens throughout the Asia/Pacific region.

This study has highlighted the usefulness of the MLST technique for monitoring the global spread of microorganisms. Undoubtably, this technique is currently the most satisfactory for such analysis, and is likely to grow in popularity as an epidemiological tool. The major limitation of this methodology, however, is its expense. A recent report by Sá-Leão and colleagues estimated the cost of typing one isolate by MLST as US$120 as opposed to US$2.50 by PFGE (Sá-Leão et al., 2001). The approach adopted in this study, typing isolate collections by PFGE or similar method, and selecting representative isolates for MLST analysis, seems an effective compromise.

7.2.2 Antibiotic resistance mechanisms

7.2.2.1 β-lactam resistance mechanism

The variable resistance to β-lactams by the Korea^{19F}-14 clone, and the reasonably homogenous genetic background (as assessed by PFGE) provided an opportunity to examine the relative contribution of individual *pbp* genes in resistance. Although, significant information was gained through examination of the nucleotide sequence of these genes, the data would have been more conclusive if interpreted with the penicillin binding affinities of individual PBPs. The data obtained from labelling with Bocillin-FL was limited, and of little use. Had time permitted, repeating the experiment with radioactively labelled penicillin would have been desirable.

Transformation of sensitive strain Rx1, with individual *pbp* genes of a resistant pneumococcal isolate would also have been desirable, and is an experiment that remains to be performed.

No attempt was made to identify non-*pbp* genes involved in β-lactam resistance of the Korea^{19F}-14 clone. As recently several genes have been identified which may also contribute to β-lactam resistance in the pneumococcus, this also warrants further exploration. In particular, the MurM enzyme involved in the biosynthesis of branched-stem cell wall muropeptides (Filipe et al., 2000a) could potentially be important in resistance (Filipe et al., 2000b, Smith and Klugman, 2001). Investigation of the *murMN* operon in the Korea^{19F}-14 clone may provide an explanation for the bimodal distribution of cephalosporin resistance observed in New Zealand.

7.2.2.2 Macrolide resistance mechanism

The Korea^{19F}-14 clone in New Zealand was found to contain two macrolide resistance genes. This was a novel finding, the only comparable report coming from South Africa (McGee et al., 2001a). Southern hybridisation of probes specific for the erythromycin resistance genes indicated they were located in the same ~5% of the genome. Further investigation is required to ascertain what elements (e.g., transposons) these genes are associated with, and if the two genes are linked via a composite genetic element. Furthermore, investigation into the macrolide resistance phenotype could be further investigated; are the resistance genes induced or constitutively expressed? is the Korea^{19F}-14 strain resistant to streptogramin B compounds, as is expected of the MLS_B phenotype?

7.2.2.3 Resistance determinants of other antibiotics

Study of macrolide resistance in the New Zealand major multi-resistant clone facilitated the finding that it harbours two different genes encoding macrolide resistance. This clone is also resistant to tetracycline and co-trimoxazole; however, the mechanisms of resistance have not been explored in this clone. Molecular studies of the mechanisms of resistance to these drugs, and others (e.g., chloramphenicol and fluoroquinolones), may reveal other novel genotypes.

7.2.2.4 Mechanisms of emerging resistance phenotypes

The rapid emergence of multi-drug resistant pneumococci during the 1980s is a stern reminder that one cannot be complacent about antibiotic resistance. Currently there are pneumococcal strains in the community resistant to all available antibiotics, save vancomycin; it would be naïve to presume that resistance could not develop to the glycopeptides and other modern antimicrobial agents. The *vanB* gene, conferring resistance to vancomycin, has been identified

in the genus *Streptococcus*, and was transferable, *in vitro*, to other species (Poyart et al., 1997). Although resistance has not yet been described in the pneumococcus, vancomycin tolerance has been observed (Gilmore and Hoch, 1999, Novak et al., 1999). This suggests that the "last line of defence" may soon have lost much of its effectiveness. Consequently it is important that pneumococci are screened for resistance to glycopeptides, and other new antimicrobials (described in Chapter 1), and that potential resistance mechanisms are investigated.

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Appendix I

Bacterial Isolate Descriptions

Table A.I. i Descriptions of Christchurch isolates of *S. pneumoniae* included in the current study.

Pen, penicillin; Ctx, cefotaxime (both determined by Etest); ERY, erythromycin, SXT, co-trimoxazole, TET, tetracycline, CHL, chloramphenicol

Isolate code	Date spec. recovered	Patient data			*pbp* RFLP		*Sma*I		MIC (μg/ml)		
		Age	Sex	Site	*2b*	*2x*	Sero	PFGE	Pen	Cef	Resistance Profile
Sp001	25-Aug-97	1	F	Ear	B	B	23F	2B	2	1	SXT, ERY, CHL
Sp002	26-Aug-97	83	M	Nose	A	A	19F	1A	4	1	SXT, ERY, TET
Sp003	02-Sep-97	1	M	Adenoid	A	A	19F	1A	2	8	SXT, ERY, TET
Sp004	03-Sep-97	69	F	Sputum	B	B	23F	2F	2	1	SXT, ERY, TET, CHL
Sp005	04-Sep-97	23	M	Sputum	G	G	--	9A	1	1	ERY, TET
Sp006	08-Sep-97	3	M	Ear	B	B	--	3A	1	1	SXT
Sp007	09-Sep-97	48	M	Sputum	B	B	19F	10B	4	2	SXT, ERY, TET, CHL
Sp008	11-Sep-97	1	F	Ear	B	B	23F	2C	2	1	SXT, ERY, CHL
Sp009	12-Sep-97	2 mth	M	Eye	B	B	9	3E	2	1	SXT
Sp010	15-Sep-97	4	F	Nose	B	B	23F	2K	2	1	SXT, ERY, TET, CHL
Sp011	16-Sep-97	4	M	Throat	O	I	--	4	1	1	ERY, TET
Sp012	24-Sep-97	1	M	Eye	B	B	19F	10B	2	1	SXT, ERY, TET, CHL
Sp013	30-Sep-97	4		Adenoid	A	A	19	1A	4	8	SXT, ERY, TET
Sp014	30-Sep-97	7	F	Pus	B	B	23F	2E	4	2	SXT, ERY, TET, CHL
Sp015	01-Oct-97	60	M	Sputum	B	B	6	8	2	1	SXT, ERY, TET, CHL
Sp016	01-Oct-97	11 mth	M	Ear	A	A	19	1A	2	1	SXT, ERY, TET
Sp017	01-Oct-97	68	M	Sputum	B	B	19	10A	2	2	ERY, TET, CHL
Sp018	03-Oct-97	73	F	Eye	D	I	--	11F	0.12	0.12	SXT, ERY
Sp019	06-Oct-97	1	F	Eye	A	B	19	1A	4	4	SXT, ERY, TET
Sp020	06-Oct-97	1	M	Ear	B	A	23F	2A	2	1	SXT, ERY, CHL
Sp021	13-Oct-97	1	M	Ear	A	A	--	1A	4	8	SXT, ERY, TET
Sp022	14-Oct-97	41	F	R. Antrum	A	A	19	1A	4	2	SXT, ERY, TET
Sp023	21-Oct-97	3	M	Adenoid	D	I	--	11A	0.12	0.12	SXT, ERY, TET
Sp024	21-Oct-97	5	F	Eye	D	I	--	11B	0.12	0.12	SXT, ERY, TET
Sp025	30-Oct-97	1	F	Throat	A	A	19	1A	4	2	SXT, ERY, TET
Sp026	30-Oct-97	1	F	Throat	A	A	--	1A	2	1	SXT, ERY, TET
Sp027	31-Oct-97	65	F	Sputum	G	G	19	9A	2	1	ERY, TET
Sp028	04-Nov-97	1	F	Pus	A	B	19	1C	4	8	SXT, ERY, TET
Sp029	07-Nov-97	1	F	Sputum	G	G	19	9A	2	1	ERY, TET
Sp030	10-Nov-97	4	F	Skin	B	A	23F	2H	2	2	SXT, ERY, TET, CHL
Sp031	11-Nov-97	5	M	Adenoid	A	A	19	1A	4	2	SXT, ERY, TET
Sp032	12-Nov-97	69	M	Sputum	B	B	19	10A	4	4	SXT, ERY, TET, CHL

Isolate code	Date spec. recovered	Patient data			*pbp* RFLP		*SmaI* MIC (μg/ml)				
		Age	Sex	Site	*2b*	*2x*	Sero	PFGE	Pen	Cef	Resistance Profile
Sp033	13-Nov-97	1	M	Adenoid	B	B	--	2J	1	0.5	SXT, TET, CHL
Sp035	19-Nov-97	1	M	Eye	B	B	23F	2A	4	2	SXT, ERY, TET, CHL
Sp036	19-Nov-97	3	M	Ear	E	L	--	14	0.12	0.12	
Sp038	21-Nov-97	1	F	Ear	A	A	19	1A	4	1	SXT, ERY, TET
Sp039	24-Nov-97	16 days	M	Eye	A	A	--	1A	4	1	SXT, ERY, TET
Sp040	25-Nov-97	27	F	Eye	A	B	nt	15	2	1	ERY
Sp041	28-Nov-97	6 mth	F	Ear	B	B	--	3A	1	ND	SXT
Sp042	02-Dec-97	69	M	Sputum	A	A	19	1A	4	8	SXT, ERY, TET
Sp043	05-Nov-97	3 mth	F	Eye	F	I	--	5A	1	1	SXT
Sp044	08-Dec-97	2	M	Eye	D	I	nt	11J	0.12	0.12	SXT, ERY, TET
Sp045	09-Dec-97	3	M	Adenoid	B	B	23F	2G	2	1	SXT, ERY, CHL
Sp046	09-Dec-97	6	M	Throat	B	B	14	3B	1	1	
Sp047	12-Dec-97	9 mth	F	Eye	E	L	--	16	0.12	0.06	SXT
Sp051	08-Jan-98	4	M	Eye	A	A	--	1A	4	1	SXT, ERY, TET
Sp055	14-Jan-98	5	F	Eye	D	I	--	11C	0.12	0.12	SXT, ERY, TET
Sp056	14-Jan-98	3	F	Eye	D	I	--	11C	0.12	0.12	SXT, ERY, TET
Sp057	16-Jan-98	76	M	Sputum	A	A	--	1A	2	1	SXT, ERY, TET
Sp058	19-Jan-98	2	M	Eye	D	I	nt	11D	0.12	0.12	SXT, ERY, TET
Sp059	20-Jan-98	70	M	Sputum	A	A	19	1A	4	16	SXT, ERY, TET
Sp061	21-Jan-98	2	M	Adenoid	A	A	19	1A	2	2	SXT, ERY, TET
Sp062	21-Jan-98	1	F	Ear	H	J	--	12A	0.12	0.06	SXT
Sp069	04-Feb-98	4	F	Eye	D	G	--	11A	0.12	0.12	SXT, ERY, TET
Sp076	18-Feb-98	2	F	Nose	G	G	--	9B	2	1	SXT, ERY, TET
Sp077	23-Feb-98	7	F	Eye	D	I	--	11D	0.12	0.12	SXT, ERY, TET
Sp078	24-Feb-98	8	F	Eye	D	I	--	11D	0.12	0.12	SXT, ERY, TET
Sp079	24-Feb-98	12	M	Ear	G	H	--	2M	1	1	SXT, ERY, TET, CHL
Sp081	25-Feb-98	4	F	Eye	M	E	--	6	1	ND	
Sp082	25-Feb-98	15	F	Eye	D	I	--	11D	0.12	0.12	SXT, ERY, TET
Sp089	11-Mar-98	70	M	Sputum	A	A	--	1A	2	4	SXT, ERY, TET
Sp091	16-Mar-98	?	M	Eye	D	I	--	11C	0.12	0.12	SXT, ERY, TET
Sp096	19-Mar-98	10 mth	F	Eye	B	B	--	7	2	0.5	SXT, TET, CHL
Sp101	24-Mar-98	18	F	Eye	D	I	--	11C	0.12	0.12	SXT, ERY, TET
Sp105	26-Mar-98	90	M	Sputum	B	B	23F	2A	4	2	SXT, ERY, CHL
Sp110	02-Apr-98	84	M	Sputum	A	A	--	1A	4	2	SXT, ERY, TET
Sp115	08-Apr-98	70	M	Sputum	A	A	--	1A	4	8	SXT, ERY, TET
Sp119	16-Apr-98	70	M	Sputum	A	A	--	1A	2	4	SXT, ERY, TET
Sp120	17-Apr-98	70	F	Ear	B	B	--	2D	2	1	SXT, ERY, CHL
Sp122	11-Apr-98	1	F	Ear	B	B	--	3B	1	ND	SXT
Sp133	01-May-98	33	F	Nose	A	A	--	1A	4	8	SXT, ERY, TET
Sp138	07-May-98	8 mth	M	Ear	A	A	--	1A	2	1	SXT, ERY, TET
Sp145	18-May-98	1	F	Ear	A	A	--	1A	2	1	SXT, ERY, TET
Sp147	16-May-98	1	F	Ear	A	A	--	1A	2	1	SXT, ERY, TET
Sp150	20-May-98	1	M	Eye	B	B	--	2G	2	1	SXT, ERY, TET, CHL
Sp160	02-Jun-98	1	M	Ear	A	A	--	1A	2	1	SXT, ERY, TET
Sp161	02-Jun-98	2	F	Adenoid	B	B	--	3D	1	0.5	SXT
Sp162	02-Jun-98	67	M	Sputum	B	B	--	2A	2	1	SXT, ERY, CHL
Sp168	04-Jun-98	25	F	Sputum	A	A	--	1A	4	1	SXT, ERY, TET
Sp171	08-Jun-98	37	M	Sputum	A	A	--	1A	2	8	SXT, ERY, TET

Isolate code	Date spec. recovered	Patient data			*pbp* RFLP		*Sma*I		MIC (μg/ml)		
		Age	Sex	Site	*2b*	*2x*	Sero	PFGE	Pen	Cef	Resistance Profile
Sp174	10-Jun-98	2	F	Nose	A	A	--	1A	4	1	SXT, ERY, TET
Sp177	12-Jun-98	43	F	Nose	A	A	19F	1A	2	1	SXT, ERY, TET
Sp180	12-Jun-98	4	M	Eye	A	A	--	1A	2	2	SXT, ERY, TET
Sp182	18-Jun-98	37	F	Ear	A	A	19F	1A	4	4	SXT, ERY, TET
Sp183	17-Jun-98	2	M	Ear	A	A	--	1A	4	1	SXT, ERY, TET
Sp191	29-Jun-98	13	F	Eye	D	I	--	11I	0.12	0.12	SXT, ERY, TET
Sp192	29-Jun-98	11	F	Eye	D	I	--	11D	0.12	0.12	SXT, ERY, TET
Sp194	30-Jun-98	1	F	Ear	A	B	--	1A	4	0.5	SXT, ERY, TET
Sp198	01-Jul-98	71	M	Sputum	A	A	--	1A	4	8	SXT, ERY, TET
Sp202	02-Jul-98	85	F	Ear	A	A	--	1A	2	1	SXT, ERY, TET
Sp206	06-Jul-98	75	M	Sputum	B	B	--	3B	1	2	
Sp211	09-Jul-98	52	F	Nose	B	B	--	3C	0.5	ND	SXT
Sp212	09-Jul-98	33	F	Nose	B	B	--	2A	4	1	SXT, ERY, CHL
Sp214	10-Jul-98	2	M	Ear	B	B	--	3A	4	1	
Sp217	13-Jul-98	81	F	Sputum	B	B	--	2E	2	1	SXT, TET, CHL
Sp221	10-Jul-98	69	M	Sputum	B	B	--	1D	2	2	SXT, ERY, TET
Sp226	16-Jul-98	2	M	Nose	A	A	--	1A	4	8	SXT, ERY, TET
Sp228	17-Jul-98	11	M	Eye	D	I	--	11J	0.12	0.12	
Sp237	23-Jul-98	2	F	Ear	A	A	--	1A	8	8	SXT, ERY, TET
Sp239	23-Jul-98	32	M	Sputum	B	B	--	3A	2	2	
Sp240	24-Jul-97	81	F	Sputum	B	B	--	2A	4	1	SXT, ERY, CHL
Sp242	27-Jul-98	?	F	Ear	L	O	--	5B	0.5	ND	SXT
Sp243	27-Jul-98	43	M	Sputum	A	B	--	1A	4	2	SXT, ERY, TET
Sp247	29-Jul-98	11 mth	F	Eye	A	A	--	1A	2	0.5	SXT, ERY, TET
Sp249	04-Aug-98	5	M	Adenoid	I	L	--	12B	0.12	0.06	SXT
Sp254	06-Aug-98	5	F	Ear	B	B	--	3B	1	ND	SXT
Sp256	07-Aug-98	9 mth	M	Ear	A	A	--	1A	4	8	SXT, ERY, TET
Sp257	07-Aug-98	6	F	Eye	D	I	--	11D	0.12	0.12	SXT, ERY, TET
Sp258	07-Aug-98	2	M	Ear	A	A	--	1A	4	2	SXT, ERY, TET
Sp259	07-Aug-98	1	M	Nose	B	B	--	3A	1	ND	SXT
Sp262	10-Aug-98	2	M	Ear	A	A	--	1A	4	8	SXT, ERY, TET
Sp269	15-Aug-98	12	M	Ear	A	A	--	1B	4	2	SXT, ERY, TET
Sp272	17-Aug-98	1	M	Ear	A	B	--	1A	4	1	SXT, ERY, TET
Sp273	18-Aug-98	2	M	Ear	A	A	--	1A	4	8	SXT, ERY, TET
Sp280	25-Aug-98	6	M	Ear	B	B	--	3C	1	0.5	SXT
Sp281	26-Aug-98	2	M	Ear	A	A	--	1A	4	8	SXT, ERY, TET
Sp284	27-Aug-98	4	M	Nose	A	A	--	1A	2	4	SXT, ERY, TET
Sp288	20-Jul-98	69	M	Sputum	A	A	--	1A	2	2	SXT, ERY, TET
Sp289	19-Nov-97	4	F	Nose	B	B	--	3B	1	ND	SXT
Sp303	09-Sep-98	2 wk	M	Eye	A	A	--	1A	8	1	SXT, ERY, TET
Sp304	10-Sep-98	34	F	Pus	B	B	--	3B	4	1	ERY, TET
Sp306	11-Sep-98	2	F	Ear	A	A	--	1A	2	1	SXT, ERY, TET
Sp307	14-Sep-98	7 mth	M	Ear	A	A	--	1A	4	8	SXT, ERY, TET
Sp312	17-Sep-98	6 mth	M	Eye	A	A	--	1A	4	1	SXT, ERY, TET
Sp324	25-Sep-98	3 mth	M	Eye	B	B	--	2I	4	1	SXT, ERY, TET, CHL
Sp330	02-Oct-98	1 wk	F	Eye	B	B	--	3C	0.5	ND	SXT
Sp332	05-Oct-98	14	M	Eye	A	A	--	1A	4	1	SXT, ERY, TET
Sp338	08-Oct-98	43	F	Nose	A	A	--	1A	2	2	SXT, ERY, TET

Isolate code	Date spec. recovered	Patient data			*pbp* RFLP		*SmaI*		MIC (µg/ml)		
		Age	Sex	Site	*2b*	*2x*	Sero	PFGE	Pen	Cef	Resistance Profile
Sp341	13-Oct-98	1	M	Eye	A	B	--	1A	1	2	SXT, ERY, TET
Sp342	13-Oct-98	45	M	Eye	D	I	--	11E	0.12	0.12	SXT, ERY, TET
Sp349	23-Oct-98	56	F	Ethmoid	A	A	--	1A	4	1	SXT, ERY, TET
Sp350	23-Oct-98	1	M	Ear	A	A	--	1A	4	2	SXT, ERY, TET
Sp351	27-Oct-98	18	F	Eye	D	I	--	11E	0.12	0.12	SXT, ERY, TET
Sp353	29-Oct-98	2	F	Eye	A	A	--	1A	2	1	SXT, ERY, TET
Sp358	04-Nov-98	1	M	Eye	A	A	--	1A	2	4	SXT, ERY, TET
Sp367	12-Nov-98	2 wk	F	Eye	A	A	--	1A	4	8	SXT, ERY, TET
Sp372	20-Nov-98	62	M	Sputum	A	A	--	1E	4	1	SXT, ERY, TET
Sp377	27-Nov-98	3	F	Eye	B	B	--	2N	2	1	SXT, TET, CHL
Sp378	27-Nov-98	41	M	Sputum	B	B	--	2G	2	2	SXT, ERY, TET, CHL
Sp380	30-Nov-98	6 wk	F	Eye	A	A	--	1A	4	2	SXT, ERY, TET
Sp383	01-Dec-98	5	M	Eye	D	I	--	11D	0.12	0.06	SXT, ERY, TET
Sp385	03-Dec-98	2	M	Ear	B	B	--	3E	0.5	0.5	SXT
Sp388	11-Dec-98	1	F	Ear	A	A	--	1A	4	2	SXT, ERY, TET
Sp401	14-Dec-98	9 mth	F	Ear	A	A	--	1A	4	8	SXT, ERY, TET
Sp405	15-Dec-98	1	M	Nose	A	A	--	1A	2	8	SXT, ERY, TET
Sp411	19-Dec-98	53	F	Ear	A	A	--	1A	2	1	SXT, ERY, TET
Sp412	22-Dec-98	7	M	Adenoid	B	B	--	3E	1	1	
Sp413	22-Dec-98	1	F	Ear	A	B	--	1A	2	1	SXT, ERY, TET
Sp414	16-Feb-99	6	F	Eye	D	I	--	11C	0.12	0.12	SXT, ERY, TET
Sp415	05-Mar-99	2	F	Nose	A	A	--	1A	4	1	SXT, ERY, TET
Sp416	05-Mar-99	2	F	Nose	A	A	--	1A	4	1	SXT, ERY, TET
Sp417	05-Mar-99	91	M	Sputum	B	B	--	2A	4	2	SXT, ERY, CHL
Sp418	08-Mar-99	1	F	Ear	B	B	--	2A	2	1	SXT, ERY, CHL
Sp419	23-Mar-99	2	M	Nose	A	A	--	1A	4	16	SXT, ERY, TET
Sp421	26-Mar-99	7 mth	M	Ear	A	A	--	1A	8	16	SXT, ERY, TET
Sp422	26-Mar-99	56	F	Sputum	B	B	--	3A	2	1	SXT
Sp423	22-Mar-99	1	F	Ear	I	L	--	17	0.12	0.06	SXT
Sp425	08-Apr-99	41	F	SW	B	B	--	3C	1	1	SXT
Sp426	09-Apr-99	40	F	Eye	D	I	--	11D	0.12	0.12	SXT, ERY, TET
Sp429	20-Apr-99	2	M	Ear	B	B	--	3D	1	ND	SXT
Sp431	28-Apr-99	16	F	Sputum	A	B	--	18	1	1	SXT, ERY, TET
Sp432	06-May-99	2 mth	F	Eye	B	B	--	3D	1	0.5	SXT
Sp433	10-May-99	8 mth	F	Eye	A	A	--	19	2	0.5	SXT, ERY, TET
Sp436	16-Oct-00	76	F	Sputum	G	D	--	2M	0.5	0.25	SXT, ERY, TET, CHL
Sp437	17-Oct-00	87	F	Sputum	B	B	--	2A	1	1	SXT, ERY, TET, CHL
Sp438	17-Oct-00	44	F	Sputum	A	A	--	1A	4	8	SXT, ERY, TET
Sp439	26-Mar-01	3	F	Ear	A	B	--	1A	1	1	SXT, ERY, TET
Sp440	04-Apr-01	30	F	Sputum	A	A	--	1A	1	0.5	SXT, ERY, TET
Sp441	09-Apr-01	1	F	Nasal	C	B	--	25	0.12	0.12	SXT, ERY, TET
Sp443	21-Apr-01	1	F	Ear	A	A	--	1A	1	2	SXT, ERY, TET
Sp444	01-May-01	76	F	Eye	A	A	--	1A	1	0.5	SXT, ERY, TET
Sp445	03-May-01	6 wk	F	Eye	B	B	--	20	1	ND	SXT
Sp446	20-May-01	7 mth	M	Ear	A	A	--	1A	1	1	SXT, ERY, TET
Sp447	05-Jun-01	1	M	Ear	A	B	--	1A	1	1	SXT, ERY, TET
Sp449	07-Jun-01	52	M	Ethmoid	B	B	--	3E	1	ND	SXT
Sp450	11-Jun-01	2	?	Nasal	A	A	--	1A	2	1	SXT, ERY, TET

Isolate code	Date spec. recovered	Patient data			*pbp* RFLP		*Sma*I		MIC (μg/ml)		
		Age	Sex	Site	*2b*	*2x*	Sero	PFGE	Pen	Cef	Resistance Profile
Sp451	12-Jun-01	2	F	Ear	B	D	--	21	0.12	0.12	ERY
Sp452	20-Jun-01	20	M	Eye	D	I	--	11G	0.12	0.12	SXT, ERY, TET
Sp453	20-Jun-01	1	F	Ear	A	B	--	1A	2	1	SXT, ERY, TET
Sp454	20-Jun-01	1	F	Ear	E	B	--	13	0.12	0.5	ERY, TET
Sp456	26-Jun-01	6 mth	F	Eye	A	A	--	1A	1	2	SXT, ERY, TET
Sp457	27-Jun-01	83	F	Sputum	D	I	--	11H	0.25	0.25	SXT, ERY, TET
Sp458	28-Jun-01	34	F	Sputum	A	A	--	1A	1	2	SXT, ERY, TET
Sp459	29-Jun-01	54	F	Sputum	A	A	--	1A	1	1	SXT, ERY, TET
Sp460	29-Jun-01	72	M	Sputum	A	A	--	1A	1	0.5	SXT, ERY, TET
Sp461	04-Jul-01	48	M	L Ethmoid	A	A	--	1A	1	1	SXT, ERY, TET
Sp462	05-Jul-01	66	M	Sputum	A	A	--	1A	1	2	SXT, ERY, TET
Sp463	05-Jul-01	70	M	Sputum	A	A	--	1E	1	1	SXT, ERY, TET
Sp464	06-Jul-01	1	M	Ear	A	B	--	1A	2	1	SXT, ERY, TET
Sp465	06-Jul-01	3	F	Vulva	B	B	--	3D	0.5	0.5	SXT
Sp466	12-Jul-01	5 mth	F	Eye	A	A	--	1A	1	2	SXT, ERY, TET
Sp467	13-Jul-01	1	M	Ear	A	B	--	1A	2	1	SXT, ERY, TET
Sp469	23-Jul-01	2	F	Eye	N	B	--	22	1	0.5	SXT, TET, CHL
Sp470	23-Jul-01	74	M	Ear	A	L	--	23	1	ND	SXT, TET
Sp471	23-Jul-01	5	F	Ear	I	L	--	24	0.12	ND	SXT
Sp472	24-Jul-01	1	F	Ear	A	A	--	1E	1	0.5	SXT, ERY, TET
Sp473	24-Jul-01	1	F	Ear	A	A	--	1A	1	2	SXT, ERY, TET
Sp474	24-Jul-01	10 mth	F	Eye	A	A	--	1A	1	1	SXT, ERY, TET
Sp475	25-Jul-01	38	F	Eye	D	I	--	11C	0.12	0.12	SXT, ERY, TET
Sp476	25-Jul-01	2	M	Ear	A	A	--	1A	1	ND	SXT, TET

Table A.I. ii Isolate data on invasive isolates characterised by MLST.

Pen, penicillin; Ctx, cefotaxime (both determined by Etest); ERY, erythromycin, SXT, co-trimoxazole, TET, tetracycline, CHL, chloramphenicol; CSF, cerebral spinal fluid; PDF, peritoneal dialysis fluid; ACF, abdominal cavity fluid; Dashes (--) signify susceptibility to all four antibiotics, (*) resistance to tetracycline not determined, (**) resistance to tetracycline and co-trimoxazole not determined.

Isolate	Source	Region	Serotype	Pen MIC (µg/ml)	Ctx MIC (µg/ml	Resistance Profile
P92-095	Blood	Auckland	14	0.016	0.016	ERY
P92-247	Blood	Hamilton	19A	0.25	0.6	SXT
P93-060	Blood	Auckland	19F	1	0.12	--
P93-075	Blood	Auckland	9N	0.016	0.016	--
P93-116	Blood	Auckland	14	0.016	0.016	ERY
P93-216	Blood	Christchurch	19A	0.016	0.016	--
P93-241	Blood	Whakatane	6B	2	1	ERY, SXT, TET, CHL
P93-295	CSF	Palmerston North	23F	0.12	0.12	--
P93-308	Blood	Auckland	19F	0.016	0.016	TET
P93-316	Blood	Christchurch	23F	0.016	0.016	--
P93-345	Blood	Dunedin	19A	0.12	0.06	SXT
P94-001	Blood	Auckland	14	0.016	0.016	SXT
P94-008	Blood	Auckland	23F	2	1	TET, CHL
P94-015	Blood	Auckland	9V	0.03	0.016	--
P94-054	Blood	Christchurch	19F	0.016	0.016	--
P94-055	Blood	Christchurch	14	0.016	0.016	--
P94-099	Blood	Auckland	23F	2	1	SXT, TET, CHL
P94-115	Blood	Auckland	9V	0.016	0.03	--
P94-212	Blood	Auckland	23F	2	1	ERY, SXT, CHL
P94-271	CSF	Christchurch	19	0.016	0.016	--
P95-066	Blood	Christchurch	19A	0.12	0.06	SXT
P95-194	Blood	Christchurch	14	0.016	0.016	--
P95-213	Blood	Palmerston North	9V	0.016	0.016	--
P95-255	Blood	Auckland	14	0.5	0.12	--
P95-298	Blood	Auckland	9N	0.03	0.016	--
P95-347	Blood	Auckland	9V	2	1	SXT
P96-165	Blood	Rotorua	19F	2	1	ERY, SXT, TET, CHL
P96-466	Blood	Auckland	6B	2	1	ERY, SXT, TET, CHL
P97-036	Blood	Whangarei	14	2	1	ERY, SXT, TET, CHL
P97-076	Blood	Auckland	19F	2	1	ERY*
P97-083	Blood	Auckland	23F	2	1	CHL*
P97-291	CSF	Dunedin	19F	4	2	ERY, SXT*
P97-350	Blood	Auckland	23F	2	1	ERY, SXT, CHL*
A97-359	CSF	Dunedin	19F	4	1	ERY, SXT, TET
P97-377	Blood	Auckland	9V	2	1	--*
P97-552	Blood	Auckland	9V	2	1	--*
P97-626	Blood	Christchurch	19F	4	8	ERY, SXT*
P98-120	PDF	Hamilton	19F	2	0.5	ERY**
P98-122	ACF	Hamilton	23F	2	1	CHL**
P98-136	CSF	Wellington	23F	2	1	CHL**
P98-206	Blood	Dunedin	9V	2	1	--**
P98-263	Blood	Hawera	9V	2	0.5	--**

Isolate	Source	Region	Serotype	Pen MIC (μg/ml)	Ctx MIC (μg/ml	Resistance Profile
P98-283	Blood	Auckland	19F	4	2	ERY, CHL**
P98-286	Blood	Christchurch	14	2	1	--**
P98-299	Blood	Christchurch	23F	2	1	ERY, CHL**
P98-356	Blood	Whangarei	9V	2	1	--**
P98-475	Blood	Auckland	23F	2	1	ERY, CHL**
P98-555	Blood	Christchurch	14	2	1	--**
A99-074	CSF	Auckland	23F	2	4	ERY, CHL

Table A.I. iii Isolate data on non-invasive isolates characterised by MLST.

Pen, penicillin; Ctx, cefotaxime (both determined by Etest), ERY, erythromycin, SXT, co-trimoxazole, TET, tetracycline, CHL, chloramphenicol.

Isolate	Source	Region	Serotype	Pen MIC (μg/ml)	Ctx MIC (μg/ml)	Resistance Profile
A93-380	Nasopharynx	Auckland	19F	2	1	ERY, SXT, TET
A93-431	Sputum	Wellington	19F	2	1	ERY, SXT, TET
A93-612	Ear	Auckland	19F	4	1	ERY, SXT, TET
A94-993	Sputum	Christchurch	19F	2	1	ERY, TET
A95-061	Eye	Christchurch	19F	2	1	ERY, SXT, TET
A95-109	Ear	Wanganui	19F	4	1	ERY, SXT
A95-176	Ear	Christchurch	19F	4	2	ERY, SXT, TET
A95-235	Sputum	Christchurch	19F	2	1	ERY, SXT, TET
A96-755	Ear	Christchurch	19F	2	1	ERY, SXT, TET
A97-006	Ear	Christchurch	19F	2	1	ERY, SXT, TET
A97-066	Ear	Hastings	19F	2	1	ERY, TET
A97-503	Ear	Wellington	19F	2	1	ERY, SXT, TET
A97-550	Eye	Hamilton	23F	2	1	ERY, SXT, CHL
A97-635	Eye	Palmerston North	19F	4	2	ERY, SXT, TET
A97-724	Ear	Palmerston North	19F	8	32	ERY, SXT, TET
A98-370	Sputum	Christchurch	19F	4	1	ERY, SXT, TET
A98-441	Ear	Christchurch	19F	4	0.5	ERY, SXT, TET
A98-537	Sputum	Invercargill	19F	0.5	16	ERY, SXT, TET
A98-619	Ear	Christchurch	19F	4	1	ERY, SXT, TET
A99-539	Eye	Christchurch	19F	4	8	ERY, SXT, TET
A99-909	Eye	Palmerston North	14	4	8	ERY, SXT, TET
A99-1014	Sputum	Hamilton	19F	4	4	ERY, SXT, TET, CHL
A00-058	Eye	Whangarei	19F	4	8	ERY, SXT, TET, CHL
A00-499	Sputum	Palmerston North	19F	4	8	ERY, SXT, TET
A00-716	Sputum	Christchurch	19F	8	16	ERY, SXT, CHL

Appendix II

Media

II.i General media

Unless otherwise stated, media were sterilised by autoclaving for 20 min at 121°C at 103.4 kPa. Solutions that were unstable at this temperature were filter sterilised by passing through a 0.22 µm filter and added to sterile media. Solid media was stored at 4°C while broths were stored at ambient temperature.

Tryptic Soya Agar (TSA) + 5% Sheep Blood

Typical Formula	Per litre
Pancreatic digest of casein	15 g
Papaic digest of soyabean meal	5 g
NaCl	5 g
Bacteriological agar	15 g
Defibrinated sheep blood	50 ml

Half volumes of the above was mixed with 475 ml of dH_2O and sterilised by autoclaving. Agar was cooled to 50°C before addition of 25 ml defibrinated sheep blood. Components were thoroughly mixed and dispensed into sterile petri dishes.

Mueller Hinton + 5% Sheep Blood Agar

Typical Formula	Per litre
Acid digest of casein	17.5 g
Beef extract	2.0 g
Starch	1.5 g
Bacteriological agar	17 g
Defibrinated sheep blood	50 ml

19 g of the agar base (Remel, Lexena, KS) was mixed with 475 ml of dH_2O and sterilised by autoclaving. Agar was cooled to 50°C before addition of 25 ml defibrinated sheep blood. Components were thoroughly mixed and dispensed into sterile petri dishes.

Brain Heart Infusion (BHI) Broth

Typical Formula	Per litre
Beef heart infusion	25 g
Calf brain infusion	20 g
Protease peptone	10 g
NaCl	5 g
Na_2HPO_4:$12H_2O$	2.5 g
Glucose	2 g

19.0 g of broth base (Oxoid, Hampshire, England) was mixed with 500 ml of dH_2O and sterilised by autoclaving.

Todd Hewitt (TH) Broth

Typical Formula	Per litre
Infusion from 450 g fat free minced meat	10 g
Tryptone	20 g
Sodium bicarbonate	2 g
NaCl	2 g
Na_2HPO_4:$12H_2O$	0.4 g

17 g of broth base (Oxoid, Hampshire, England) was mixed with 500 ml of dH_2O and sterilised by autoclaving. Where stated in the text, TH broth was supplemented with 0.5% (w/v) yeast extract, added prior to autoclaving.

Appendix III

Buffers and Solutions

Buffers and solutions requiring sterilisation were autoclaved for 20 min at 121°C at 103.4 kPa or filter sterilised by passing through a 0.22 μm filter. Unless stated otherwise, all buffers and solutions were stored at ambient temperature.

III.i General stock solutions and buffers

0.9% Saline

	Per litre
0.15 M NaCl	9.0 g

Dissolved in dH_2O to a final volume of 1 litre.

Phosphate Buffered Saline

	Per litre
145 mM NaCl	8.5 g
1.7 mM KH_2PO_4	0.3 g
4.2 mM Na_2HPO_4	0.6 g

Dissolved in dH_2O to a final volume of 1 litre, pH adjusted to 7.4.

Phosphate buffer, pH 6.0

	Per litre
50 mM KH_2PO_4	6.8 g
5.7 mM NaOH	0.23 g

Dissolved in dH_2O to a final volume of 1 litre, pH checked.

10% Sodium Dodecyl Sulphate (SDS)

	Per litre
SDS	100 g

Dissolved in dH_2O at 68°C to a final volume of 1 L. pH was adjusted to 7.2 with concentrated hydrochloric acid.

III.ii Standard agarose electrophoresis buffers & solutions

50× TAE

	Per litre
50 mM Tris base	242 g
0.11% v/v glacial acetic acid	57.1 ml
1 mM EDTA (pH 8.0)	46.5 g

Made up to 1 L in dH_2O and pH adjusted to 8.0.

1× TAE

20 ml of 50× TAE made up to 1 L with dH_2O.

6× DNA Loading Dye for Agarose Gel Electrophoresis

50% (v/v) Glycerol

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene cyanol

Made up to 10 ml in dH_2O.

III.iii Chromosomal DNA Purification Solutions

1× TE Buffer

10 mM Tris-HCl

1 mM EDTA

50 ml of 20× TE buffer made up to 1 L with dH_2O.

GES Lysis Solution

	Per 100 ml
Guanidium thiocyanate	60 g
0.1 M EDTA (pH 8.0)	20 ml (0.5 M stock)
dH_2O	20 ml
10% v/v N-lauroyl sarcosine	5 ml

Guanidium thiocyanate and dH_2O heated at 65°C and mixed until dissolved. 10% N-lauroyl sarcosine added after cooling. Made up to 100 ml with dH_2O and passed through a 0.45 μm filter.

7.5 M Ammonium Acetate

	Per litre
$NH_4C_2H_3O_2$	577.5 g

Dissolved in dH_2O to a final volume of 1 L.

24:1 Chloroform/IAA

	Per 50 ml
Chloroform	48 ml
Isoamyl alcohol (IAA)	2 ml

Mixed thoroughly and stored at 4°C in a dark glass bottle.

III.iv PFGE Buffers And Solutions

Molecular biology grade reagents and distilled water were used wherever possible for all solutions and buffers. PFGE buffers and solutions were either autoclaved at 121°C at 120 kPa or filter sterilised through a 0.22 μm filter.

III.iv.i PFGE Stock Solutions

1 M Tris-HCL

	Per 200 ml
Tris base	24.22 g

Dissolved in 150 ml dH_2O with gentle heat, pH adjusted to 7.6 by addition of concentrated HCl. Made up to final volume of 200 ml and stored at 4°C.

0.5M EDTA

	Per litre
EDTA	186.1 g
NaOH pellets	22 g

Dissolved in 800 ml dH_2O with gentle heat. pH adjusted to 8.0 by addition of more NaOH pellets. Made up to final volume of 1 L and stored at 4°C.

5 M Sodium Chloride

	Per litre
NaCl	295 g

Dissolved in 800 ml dH_2O by heating. Made up to 1 L final volume.

20× TE Buffer

	Per litre
200 mM Tris-HCl	24.22 g
2 mM EDTA	4 ml (0.5 M stock)

Dissolved in dH_2O, pH adjusted to 8.0.

10× PFG-TBE (Tris-borate-EDTA)

	Per litre
1 M Tris-HCL	121.1 g
1 M Boric Acid	61.83 g
2 mM EDTA	4 ml (0.5 M stock)

Dissolved in 800 ml dH_2O with gentle heat. Made up to 1 L and stored at 4°C.

III.iv.ii PFGE working solutions

EC Lysis Buffer

	Per 400 ml
6 mM Tris-HCl	2.4 ml (1 M stock)
1 M NaCl	80 ml (5 M stock)
100 mM EDTA	80 ml (0.5 M stock)
0.5% Brij-58	2 g
0.2% Sodium deoxycholate	0.8 g
0.5% N-Lauroyl sarcosine	2 g

Dissolved in 100 ml dH_2O with gentle heat, Made up to 400 ml and filter sterilised in 20 ml aliquots. Stored at -20°C. Before use, 1 mg/ml (final conc.) lysozyme and 20 µg/ml (final conc.) of heat-treated RNase was added.

ESP Buffer

	Per 400 ml
0.5 M EDTA	400 ml
1% N-Lauroyl sarcosine	4 g

N-Lauroyl sarcosine was added to sterile 0.5 M EDTA solution and shaken vigorously to dissolve. 0.5 mg/ml (final conc.) Proteinase K was added prior to use.

PETT IV Buffer

	Per litre
10 mM Tris-HCl (pH 7.6)	10 ml (1 M stock)
1 M NaCl	200 ml (5 M stock)

Made up to 1 L in dH_2O and stored at 4°.C.

1× TE Buffer

	Per 500 ml
50× TE stock buffer	25 ml

Made up to 500 ml in dH_2O and stored at 4°C.

0.5× PFG-TBE

	Per 2 L
10× PFG-TBE stock	100 ml

Made up to 2 L in dH_2O immediately prior to use.

III.v Southern Hybridisation Buffers and Solutions

Depurination Solution

	Per litre
0.2 M HCl	17.2 ml

Prepared using concentrated HCl diluted with dH_2O.

Denaturation Solution	Per litre
0.5 M NaOH	20 g
1.5 M NaCl	87.7 g

Dissolved in dH_2O.

Neutralisation Solution	Per litre
0.5 M Tris-HCl (pH 7.0)	76.9 g
3 M NaCl	175.5 g

Dissolved in dH_2O, adjusted to pH of 7.0.

20× SSC (Standard Saline Citrate)	Per litre
3 M NaCl	175 g
0.3 M Trisodium citrate	88 g

Dissolved in dH_2O, adjusted to pH of 7.0 with citric acid.

Transfer Solution

20× SSC, pH 7.0

DIG Buffers and Solutions

1 M Maleic Acid

	Per litre
Maleic acid	116.1 g

Made up to 1 L in dH_2O and stored at ambient temperature.

Maleic Acid Buffer

	Per litre
0.1 M maleic acid	100 ml (1 M stock)
0.15 M NaCl	30 ml (5 M stock)

Adjusted to pH of 7.5 with NaOH pellets.

Washing Buffer

Maleic Acid buffer

0.3% v/v Tween 20

Blocking Solution

10% v/v blocking reagent (kit-stored in freezer)

90% v/v maleic acid buffer.

Prepared freshly for each hybridisation, heated to 37°C to dissolve

Post-Hybridisation Low Stringency Wash I

	Per litre
2× SSC	100 ml (20× stock)
0.1% SDS	10 ml (10%)

Made up to 1 L with dH_2O

Detection Buffer

	Per litre
0.1 M Tris-HCl	100 ml (1 M stock)
0.1 M NaCl	20 ml (5 M stock)

Adjusted to pH of 9.5 with NaOH pellets, made up to 1 L with dH_2O

III.vi Protein Extraction and Labelling Buffers and Solutions

Protein Extraction Buffer

	Per litre
140 mM NaCl	8.2 g
20 mM KH_2PO_4	3.5 g

Dissolved in dH_2O to a final volume of 1 litre, pH adjusted to 7.5.

III.vi.i SDS-PAGE stock solutions

1.5 M Tris-HCL (pH 8.8)

	Per 100 ml
Tris base	18.15 g

Dissolved in 75 ml dH_2O with gentle heat, pH adjusted to 8.8 by addition of concentrated HCl. Made up to final volume of 100 ml and stored at 4°C.

0.5 M Tris-HCL (pH 6.8)

	Per 100 ml
Tris base	6.05 g

Dissolved in 75 ml dH_2O with gentle heat, pH adjusted to 6.8 by addition of concentrated HCl. Made up to final volume of 100 ml and stored at 4°C.

30% w/v Acrylamide/Bis solution (37.5:1 ratio) stock (BioRad)

	Per 500 ml
Acrylamide	146.1 g
N, N'-methylene-bis-acrylamide	3.9 g

Purchased commercially from BioRad.

Ammonium persulphate solution (1ml)

10% Ammonium persulphate	0.1 g

Made in 1 ml dH_20 prior to use, stored short term at -20°C.

SDS-PAGE running buffer

	Per litre
25 mM Tris	3 g
10 mM Glycine	14.4 g
0.1 % SDS	1 g

Make up to volume with dH_2O, stored at 4°C.

Sample buffer

	Per 10 ml
0.125 M Tris	8.3 ml (1.5 M stock)
4% SDS	0.4g
5% beta-mercaptoethanol	500 µl
5% Glycerol	1 ml
0.25% (w/v) Bromophenol blue	

Made up to 10 mls dH_2O, stored in 1 ml aliquots at -20°C

III.vi.ii SDS-PAGE gel formulations

7.5% Resolving gel

	Per 10 ml
7.5% Acrylamide/Bis	2.5 ml (30% stock)
375 mM Tris-HCl (pH 8.8)	2.5 ml (1.5 M stock)
1% SDS	100 µl (10% stock)
dH_2O	4.85 ml
TEMED	5 µl
0.5% APS	50 µl (10% stock)

4.0% Stacking gel

	Per 10 ml
4.0% Acrylamide/Bis	1.3 ml (30% stock)
125 mM Tris-HCl (pH 6.8)	2.5 ml (0.5 M stock)
1% SDS	100 µl (10% stock)
dH_2O	6.10 ml
TEMED	5 µl
0.5% APS	50 µl (10% stock)

Appendix IV

β-Lactam MICs by Agar Dilution

Table A.IV. MICs of seven β-lactam antibiotics; raw data of 43 isolates of New Zealand 19F clone.
Pen, penicillin; Pip, piperacillin; Ceph, cephalothin; Cfux, cefuroxime; Ceft, cefotaxime; Mero, meropenem.

Strain #	MIC by Agar Dilution (μg/ml)							Etest MIC	
	Pen	Pip	Ceph	Cclor	Cfux	Ceft	Mero	Pen	Ceft
ATCC 49619	0.5	0.5	1	2	0.5	0.12	0.06	ND	ND
98-537	0.5	2	16	≥ 64	≥ 64	16	0.5	0.5	16
97-066	1	2	8	≥ 64	4	1	0.5	2	1
97-076	1	2	4	≥ 64	4	1	0.5	2	1
98-542	2	2	8	≥ 64	4	1	0.5	2	0.5
96-755	2	4	16	≥ 64	4	1	0.5	2	1
97-291	2	4	8	≥ 64	8	1	0.5	4	2
97-769	2	4	32	≥ 64	16	4	1	ND	ND
97-816 (Sp038)	2	4	16	≥ 64	4	1	0.5	4	1
98-120	2	4	16	≥ 64	4	1	0.5	2	0.5
98-370	2	4	8	≥ 64	4	1	0.5	4	1
98-491 (Sp202)	2	4	8	≥ 64	4	1	0.5	2	0.5
98-618 (Sp272)	2	4	16	≥ 64	8	1	1	4	1
98-619	2	4	16	≥ 64	8	1	0.5	4	1
98-740	2	4	16	≥ 64	8	1	0.5	4	1
98-779	2	4	8	≥ 64	4	1	0.5	4	1
98-997	2	4	16	≥ 64	8	1	0.5	ND	ND
00-482	4	4	32	≥ 64	32	8	0.5	8	16
00-486	4	4	32	≥ 64	32	8	0.5	4	16
00-761	4	4	16	≥ 64	32	8	1	4	16
00-903	4	4	32	≥ 64	32	8	1	4	16
95-176 (Sp042)	4	4	32	≥ 64	32	8	1	4	2
97-469	4	4	16	≥ 64	32	8	0.5	2	8
97-626	4	4	16	≥ 64	32	8	1	4	8
97-838	4	4	16	≥ 64	32	8	1	4	8
98-509	4	4	32	≥ 64	32	8	1	8	8
98-558	4	4	32	≥ 64	32	8	1	4	8
98-560 (Sp262)	4	4	32	≥ 64	32	8	1	4	8
98-653	4	4	16	≥ 64	32	8	1	4	8
98-698	4	4	16	≥ 64	8	1	0.5	2	2
98-853	4	4	32	≥ 64	32	8	1	2	4

Strain #	MIC by Agar Dilution (μg/ml)							Etest MIC	
	Pen	Pip	Ceph	Cclor	Cfux	Ceft	Mero	Pen	Ceft
98-886	4	4	16	≥ 64	32	8	1	4	8
99-1014	4	4	32	≥ 64	32	8	1	4	4
99-205 (Sp419)	4	4	16	≥ 64	32	8	1	4	16
00-716	4	8	32	≥ 64	32	8	1	8	16
00-879	4	8	32	≥ 64	32	8	1	4	16
00-942	4	8	32	≥ 64	32	8	1	4	16
97-724	4	8	32	≥ 64	32	8	1	8	32
98-097 (Sp059)	4	8	32	≥ 64	32	8	1	4	16
98-259	4	8	16	≥ 64	32	8	1	2	4
98-441 (Sp194)	4	8	16	≥ 64	8	1	1	4	0.5
99-206 (Sp421)	4	8	32	≥ 64	32	8	1	8	16
99-539	4	8	32	≥ 64	32	8	1	4	8
99-909	4	8	16	≥ 64	32	8	1	4	8